PB81-135329

Uptake, Metabolism, and Disposition of Xenobiotic Chemicals in Fish. Wisconsin Power Plant Impact Study

Medical Coll. of Wisconsin
Milwaukee

Prepared for

Environmental Research Lab.-Duluth, MN

Aug 80

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO.
EPA-600/3-80-082

2.

3. RECIPIENT'S ACCESSION NO.
PB81 155329

4. TITLE AND SUBTITLE
Uptake, Metabolism, and Disposition of Xenobiotic Chemicals in Fish
Wisconsin Power Plant Impact Study

5. REPORT DATE
August 1980 Issuing Date

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Department of Pharmacology and Toxicology
Medical College of Wisconsin
Milwaukee, Wisconsin

10. PROGRAM ELEMENT NO.
1BA820

11. CONTRACT/GRANT NO.
R803971

12. SPONSORING AGENCY NAME AND ADDRESS
Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Duluth, Minnesota 55804

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA/600/03

15. SUPPLEMENTARY NOTES

16. ABSTRACT

The effects and fate in fish of a number of chemicals, including hydrocarbons and chlorinated hydrocarbons, have been examined. The interactions between these chemicals and fish have been studied using several approaches.

The uptake an elimination of ^{14}C-labeled napthalene, 2-methylnapthalene, 1,2,4-trichlorobenzene, pentachlorophenol, and pentachloroanisole were studied. Each of these chemicals was taken up rapidly by rainbow trout. Increasing the duration of exposure to ^{14}C-napthalene or ^{14}C-2-methylnapthalene affected the elimination of ^{14}C-containing components from these fish. Activities of cytochrome P-450-related xenobiotic metabolizing enzymes in rainbow trout livers were induced. The quantities of biliary metabolites in these fish were considerably higher than those found in non-induced trout.

Piperonyl butoxide reduced levels of biliary metabolites of pentachloranisole and di-2-ethylhexyl-phthalate in trout and increased tissue levels of these chemicals. The high levels of biotransformation products of these chemicals found in fish bile during and after exposure to the chemicals in these studies support the possible use of bile sampling in pollutant-modelling programs.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Xenobiotic chemicals Uptake Metabolism Depuration Fish	Coal-fired power plant Fish	06/A

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS *(This Report)* Unclassified	21. NO. OF PAGES 157
	20. SECURITY CLASS *(This page)* Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

☆ U.S. GOVERNMENT PRINTING OFFICE: 1980--657-165/0097

United States
Environmental Protection
Agency

Environmental Research
Laboratory
Duluth MN 55804

EPA-600/3-80-082
August 1980

PB81-135329

Research and Development

EPA

Uptake, Metabolism, and Disposition of Xenobiotic Chemicals in Fish

Wisconsin Power Plant Impact Study

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NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

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EPA-600/3-80-082
August 1980

UPTAKE, METABOLISM AND DISPOSITION OF XENOBIOTIC CHEMICALS IN FISH

Wisconsin Power Plant Impact Study

by

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Grant No. R803971

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This study was conducted in cooperation with

Wisconsin Power and Light Company,
Madison Gas and Electric Company,
Wisconsin Public Service Corporation,
Wisconsin Public Service Commission, and
Wisconsin Department of Natural Resources

ENVIRONMENTAL RESEARCH LABORATORY-DULUTH
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
DULUTH, MINNESOTA 55804

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FOREWORD

The U.S. Environmental Protection Agency (EPA) was created because of increasing public and governmental concern about the dangers of pollution to the health and welfare of the American people. Polluted air, water, and land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated attack on the problem.

Research and development, the necessary first steps, involve definition of the problem, measurements of its impact, and the search for solutions. The EPA, in addition to its own laboratory and field studies, supports environmental research projects at other institutions. These projects are designed to assess and predict the effects of pollutants on ecosystems.

One such project, which the EPA has supported through its Environmental Research Laboratory in Duluth, Minnesota, is the study "The Impacts of Coal-Fired Power Plants on the Environment." This investigation, carried out by the Institute for Environmental Studies of the University of Wisconsin-Madison, in cooperation with the Wisconsin Power and Light Company, Madison Gas and Electric Company, Wisconsin Public Service Corporation, Wisconsin Public Service Commission, and Wisconsin Department of Natural Resources, is monitoring and evaluating the impacts of a new coal-fired power plant on the immediate environment.

During the next year reports from this study will be published as a series within the EPA Ecological Research Series. These reports will include topics related to chemical constituents, chemical transport mechanisms, biological effects, social and economic effects, and integration and synthesis.

This report presents results from the Hazardous Chemicals in Fish subproject. In a series of laboratory studies the investigators have explored the effects and fate in fish of various chemical compounds that are associated with fossil fuels.

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ABSTRACT

The effects and fate in fish of a number of chemicals, including hydrocarbons and chlorinated hydrocarbons, have been examined. The interactions between these chemicals and fish (generally rainbow trout) have been studied using several approaches: examination of the uptake, metabolism, and elimination of selected chemicals by fish; assessment of the effects of selected chemicals (inducing agents) on hepatic xenobiotic metabolizing enzymes (assayed *in vitro*); and studies of the effects of inducing agents on the metabolism and disposition of other chemicals *in vitro*.

The uptake and elimination of ^{14}C-labeled naphthalene, 2-methylnaphthalene, 1,2,4-tri-chlorobenzene, pentachlorophenol, and pentachloroanisole were studied. Each of these chemicals was taken up rapidly by rainbow trout. The ^{14}C derived from all compounds except pentachloroanisole was released rapidly ($t_{1/2}$ of elimination < 1 day), whereas pentachloroanisole-derived ^{14}C was released more slowly ($t_{1/2}$ about 6 days). Increasing the duration of exposure to ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene affected the elimination of ^{14}C-containing components from these fish, apparently because of a slower release rate for metabolites of these compounds which accumulated in tissues during longer exposure periods.

Activities of cytochrome P-450-related xenobiotic metabolizing enzymes in rainbow trout livers were induced (elevated by 3-methylcholanthrene-type inducers but not by phenobarbital-type inducers). When fish that were preinjected with 3-methylcholanthrene-type inducers were subsequently exposed to ^{14}C-labeled naphthalene, 2-methylnaphthalene, and trichlorobenzene, the quantities of biliary metabolites in these fish were considerably higher than those found in non-induced trout.

An inhibitor of cytochrome P-450-related xenobiotic metabolism, piperonyl butoxide, was shown to reduce levels of biliary metabolites of pentachloroanisole and di-2-ethylhexyl-phthalate in rainbow trout exposed to these chemicals and to increase tissue levels of these chemicals.

The high levels of biotransformation products of these chemicals found in fish bile during and after exposure to the chemicals in these studies support the possible use of bile sampling in pollutant-modeling programs.

Most of the funding for the research reported here was provided by the U.S. Environmental Protection Agency. Funds were also granted by the University of Wisconsin-Madison, Wisconsin Power and Light Company, Madison Gas and Electric Company, Wisconsin Public Service Corporation, and Wisconsin Public Service Commission. This report was submitted in fulfillment of

Grant No. R803971 by the Environmental Monitoring and Data Acquisition Group, Institute for Environmental Studies, University of Wisconsin-Madison, under the partial sponsorhsip of the U.S. Environmental Protection Agency. The report covers the period of July 1975-July 1978, and work was completed as of April 1979.

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ACKNOWLEDGMENT

The authors wish to acknowledge the other researchers of the Department of Pharmacology and Toxicology at the Medical College of Wisconsin who have participated in the work included in this report. These contributors include Clifford R. Elcombe, Andrew H. Glickman, Charles N. Statham, Lawerence A. Menahan, Anthony Wu, Jill Saybolt, and Susan B. Szyjka.

SECTION 1

INTRODUCTION

The operation of a coal-fired power plant presents opportunities for a variety of organic chemicals to reach the environment. When a power plant is situated adjacent to a river or a lake, some of these organic pollutants may be presented to fish inhabiting the general area of the power plant. Although the levels of these chemicals in the water may be too low to result in lethality or other obvious forms of toxicity to the fish, other more subtle interactions may occur. In fact, the accumulation of such chemicals by fish might be harmful not to fish themselves but to humans who consume them.

Many organic chemicals are likely to be taken up from the aqueous environment by fish and there may be considerable differences in the fate of these chemicals within the fish. Among the most obvious are differences in the biotransformation of these pollutants by the fish, differences in the distribution of these pollutants or their biotransformation products in the fish, and differences in the rates of elimination of these pollutants and their biotransformation products by the fish. In addition, one chemical might affect any of these parameters relative to another chemical in the fish. For example, such interactions could result from induction or inhibition of hepatic xenobiotic metabolizing enzymes.

Accordingly, the present study was designed to evaluate the uptake, biotransformation, distribution, and elimination of selected organic chemicals by fish and the ability of various chemicals to affect these parameters.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The characterization of rainbow trout liver subcellular fractions showed that cytochrome(s) P-450 and most xenobiotic metabolizing enzymes are present in the microsomal fraction, as is the case in higher animals. Although total cytochrome P-450 levels were increased only slightly by 3-methylcholanthrene-type inducers (β-naphthoflavone, Aroclors 1242 and 1254, etc.), a variety of cytochrom P450 related enzyme activities were increased as much as 40-fold. Gel electrophoresis demonstrated the appearance of a new cytochrome P-450 having a molecular witht of about 57,000 in response to these inducers.

Studies with ^{14}C-labeled compounds *in vivo* showed that naphthalene, 2-methylnaphthalene, pentachlorophenol, pentachloroanisole, trichlorobenzene, and di-2-ethylhexyl phthalate were taken up readily by fingerling rainbow trout. After exposures to aqueous naphthalene, 2-methylnaphthalene, pentachlorophenol, or trichlorobenzene for 1 day or less, the half-times for elimination from various tissues were less than 1 day. Similar exposures to pentachloroanisole resulted in half-times of elimination of about 1 week. After long-term exposures of fingerling rainbow trout to aqueous naphthalene or 2-methylnaphthalene, however, there was a slower rate of elimination of at least part of the accumulated ^{14}C, apparently due to a slower release from certain tissues of biotransformation products than of the parent chemicals.

Biotransformation products of these compounds were found in the bile of the trout exposed to each chemical. Di-2-ethylhexyl phthalate and pentachlorophenol were metabolized to the greatest extent, naphthalene and 2-methylnaphthalene somewhat less, and pentachloroanisole and trichlorobenzene the least.

β-naphthoflavone, an inducer of xenobiotic metabolism, increased the levels of biotransformation products of naphthalene, 2-methyl-naphthalene, and trichlorobenzene found in trout bile and decreased the tissue levels of the parent chemicals in rainbow trout exposed to these chemicals. Piperonyl butoxide, an inhibitor of xenobiotic metabolism, decreased the levels of biotransformation products of di-2-ethylhexyl phthalate and pentachloroanisole in trout bile and increased tissue levels of the parent chemicals in rainbow trout exposed to these chemicals.

RECOMMENDATIONS

We have found biotransformation products of the various chemicals under study in fish tissues and bile. The accumulation of such biotransformation products may be as important as the presence of the parent chemicals themselves. These biotransformation products might be more toxic than the original pollutants, and analysis for the original pollutants might miss the bulk of the pollutant-derived material. Examples of metabolic transformations that result in the formation of more toxic compounds are the formation of proximate carcinogens from precarcinogens and the oxidation of the weakly toxic parathion to the highly toxic paraoxon. An example of a chemical whose metabolites are more likely to be found than the original chemical is malathion, which is so rapidly metabolized that 24 h after exposure only hydrolysis products are found in fish tissues. Additional studies should be done in fish on the accumulation of the biotransformation products of the various organic pollutants that arise from the operation of a coal-fired power plant.

The high levels of the biotransformation products of various chemicals found in fish bile are important for another reason. The use of bile may provide a convenient method of sampling fish for determining previous exposure of the fish to pollutants. Although some pollutants (particularly lipophilic ones such as PCBs) accumulate to high levels in fish tissues, we have shown that the metabolites of a wide variety of chemicals (including relatively water soluble ones) accumulate to high levels in fish bile. In some cases these metabolites may be found in bile at high levels days after the exposure to the pollutant has been terminated. Work should continue on the development of a pollution monitoring system based on the presence of biotransformation products of organic pollutants in fish bile.

Because of the possibility that one chemical might affect the metabolism and disposition of other chemicals in the environment, long-term exposures of fish to likely environmental levels of such chemicals should be performed to permit assessment of the effects of such exposures on fish liver xenobiotic metabolizing enzymes.

In addition, studies should be undertaken to determine which organic chemicals actually reach the aqueous environment from the operation of a coal-fired power plant. Selections should be done in fish on the accumulation of the biotransformation products of the various organic pollutants that arise from the operation of a coal-fired power plant.

The high levels of the biotransformation products of various chemicals found in fish bile are important for another reason. The use of bile may provide a convenient method of sampling fish for determining previous exposure of the fish to pollutants. Although some pollutants (particularly lipophilic ones such as PCBs) accumulate to high levels in fish tissues, we have shown that the metabolites of a wide variety of chemicals (including relatively water soluble ones) accumulate to high levels in fish bile. In some cases these metabolites may be found in bile at high levels days after the exposure to the pollutant has been terminated. Work should continue on

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In addition, studies should be undertaken to determine which organic chemicals actually reach the aqueous environment from the operation of a coal-fired power plant. Selection of chemicals for the current studies with fish was based mainly on the results of laboratory studies concerning the extractability of components of coal and petroleum products into water.

SECTION 3

OVERVIEW

OBJECTIVES

Briefly stated, the objective of this project is to investigate in fish the dynamics of the uptake, biotransformation, and disposition of selected hazardous chemicals which might arise from the operation of a coal-fired power plant. In addition to simply measuring the uptake and release of various hazardous chemicals by fish, the research centers on the biotransformation and disposition of selected xenobiotic chemicals in fish and encompasses three interrelated long-term objectives. The first objective is to determine the metabolic pathways of potentially hazardous chemicals in fish and the extent to which biotransformation influences the level and persistence of these chemicals and their metabolites in fish tissues. The second objective is to determine the role of biotransformation in the protection of fish against toxic chemicals in the aquatic environment. The third objective, which is implicit in these research goals, is to evaluate the modification of biotransformation and excretion processes in fish by chemicals such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and methylene-dioxyphenyl synergists which are known to modulate xenobiotic metabolism in mammals.

The studies have been designed to investigate in fish the dynamics of the uptake, biotransformation, distribution, and elimination of selected hydrocarbons which are present in coal and petroleum and chlorinated hydrocarbons which may be used in or derived from algacide treatment of power-plant cooling systems. Data obtained from these studies will be used to evaluate a pharmacokinetic model to predict the accumulation and elimination of hazardous residues in fish exposed to spills or effluent mixing zones arising from chlorination practices.

SCOPE AND LIMITS OF THE INVESTIGATION

In order to meet the objectives of the investigation, the following studies have been initiated:

1. Short term and long term uptake, distribution, and elimination of selected organic chemicals and their biotransformation products on fish.

2. Xenobiotic metabolism by fish *in vivo*.

3. Xenobiotic metabolism by fish *in vivo*.

4. Modification of xenobiotic metabolism by fish *in vitro*.

5. Effects of such modification of xenobiotic biotransformation processes in fish on the biotransformation, disposition, and elimination of xenobiotics *in vivo*.

ORGANIC POLLUTANTS

For many years the hazards of waterborne chemicals were evaluated by relatively insensitive criteria, such as whether the pollutants were harmful to fish or other aquatic species. Experience with one class of organic chemicals, the PCBs, has shown that some species of fish can accumulate these chemicals to very high levels without apparent harm. These accumulated PCBs, however, may have harmful effects on higher animals, including man, which consume these fish. Information concerning which pollutants are accumulated by fish, where they are stored, and in what form they are stored, is germaine to rational approaches to monitoring for potentially hazardous chemicals in water.

The composition of coal and petroleum and their wide-spread uses suggest that these materials could be major contributors of organic chemicals to the aquatic environment. Many organic chemicals appear in water shaken with pulverized coal (Carlson and Caple 1979) or with various oils (Boylan and Tripp 1971, Lee et al. 1974, Larson and Weston 1976). Naphthalene and methylnaphthalenes are major constituents of these aqueous extracts. Anderson (1975) utilized dispersions of crude oil and fuel oil in water to study the uptake of petroleum constituents by several marine species. Several invertebrates accumulated naphthalene and methylnaphthalene, and killifish accumulated substantial amounts of naphthalene.

Treatment of the cooling systems of power plants to prevent the buildup of algae may contribute chlorinated organic pollutants either directly by the use of pentachlorophenol or indirectly by chlorination.

Carlson and Caple (1979) have shown that several organic chemicals are chlorinated by dilute chlorine under laboratory conditions. This process also occurs during the chlorination of drinking water and sewage. Laboratory studies of the chlorination of cooling-water concentrates under conditions simulating those used in the cooling system of an electric power-generating plant showed that 0.5% of the chlorine present became incorporated into the organics present (Jolly et al. 1976). Except for pesticides containing chlorine and PCBs, little has been done in studying chlorinated hydrocarbons in fish.

Based on the above considerations, we selected for study naphthalene, 2-methylnaphthalene, pentachlorophenol, pentachloroanisole, and 1,2,4-trichlorobenzene. Although not a potential pollutant from power plants, di-2-ethylhexylphthalate, a widespread ester pollutant, was already under study when this project began, and this work has been completed.

METABOLIC TRANSFORMATION OF XENOBIOTICS

A variety of species can modify the structures of chemicals that are not constituents of or participants in the usual metabolic pathways. A subcellular structure called the endoplasmic reticulum has the capability of enzymically catalyzing the alteration of the structures of a wide variety of organic compounds. In most animal species studied, the ability to catalyze such reactions resides mainly in the liver, but to a lesser degree the process occurs in a large variety of tissues. When liver tissue is homogenized, this activity is associated with small particles called microsomes. More specifically, this activity is due to the catalytic activity of a class of hemoproteins, the cytochrome(s) P-450, so called because of an absorption peak at about 450 nm. This enzyme activity, in general, is called monooxygenase activity because of the activation of a single oxygen atom from O_2 during the course of the reaction. Although a wide variety of reactions are catalyzed by cytochrome(s) P-450, the concern of this study is those reactions related to xenobiotic chemicals. In general, the foreign chemical is metabolized to a more polar compound that is more readily eliminated by the organism. For example, one or more hydroxyl groups may be added to a foreign chemical, increasing its water solubility and thereby increasing the likelihood of its elimination through the kidney. In addition, this process provides for the possibility of attachment to hydroxyl groups of other polar moieties such as sulfate, glucuronic acid, and glutathione, which could further facilitate excretion of the chemical via the kidney or biliary active transport systems.

In addition to facilitating excretion of the foreign chemical, the monooxygenase activity may also create a more toxic chemical. For example, the action of cytochrome(s) P-450 converts the pesticide parathion to the toxic form, paraoxon, and converts the polycyclic aromatic hydrocarbon carcinogens to the proximate carcinogens. Several chemicals, such as phenobarbital and 3-methylcholanthrene, increase (induce) the amount of cytochrome(s) P-450 and related enzymic activities following *in vivo* administration. The various inducers do not increase all enzymic activities equally, nor are the increases in enzymic activity necessarily proportional to the increase in total cytochrome(s) P-450. Several chemicals, such as piperonyl butoxide, inhibit the enzymic activity of cytochrome(s) P-450 when they are administered *in vivo* or when they are added to the microsomes *in vitro*.

Previous Studies of Xenobiotic Compounds in Fish

The biotransformation of drugs and xenobiotic substances in fish has only recently received attention, although much progress has been made in this area in mammals. Early studies concerned with biotransformation of drugs in several species of fish led to the conclusions that fish do not metabolize drugs to any extent and that they do not conjugate foreign phenols (Brodie and Maickel 1962).

The lack of need for these biotransformation processes was supported teleologically by the aqueous environment of fish and by their ability to excrete lipid soluble drugs through the gills into the sink of that environment. The implication of this concept is that an outward concentration gradient always exists for this important excretory process when compounds are injected into fish and provisions are made to maintain an adequate outward concentration gradient. The work of Maren et al. (1968) has provided evidence that lipid solubility is an important determinant of the speed of outward gill diffusion and that injected compounds which are ionized at the pH of fish blood have longer half-lives than those which are unionized. While such diffusion is important in situations involving ingested foreign compounds, a common and potentially harmful situation occurs when fish are exposed to a constant concentration of a given compound in the aqueous environment. In such an instance the gradient is inwards, and, even after the steady state has been established, no net outward gradient exists across the gill. Any protection against the offending substance must therefore be afforded by biotransformation and excretion mechanisms when and if they exist.

Several reports have shown that both freshwater and saltwater fish have the enzymes to oxidize many drugs and foreign compounds (Adamson 1967, Dewaide and Henderson 1968, Buhler and Rasmussen 1968) and that these enzymes appear to be microsomal in nature and require NADPH and O_2. The levels of these enzymes in both fish liver and kidney are species dependent but, in general, seem to be about 5-10 times lower than those found in mammals (Adamson 1967). Nitroreductase, glucuronyl transferase, and sulfotransferase have also been found in many species of fish (Dutton and Montgomery 1968, Adamson et al. 1965). *In vitro* metabolism of parathion, guthion, diazinon, and dimethylnitrosamine by fish liver has been demonstrated in several laboratories (Potter and O'Brien 1964, Murphy 1966, Hogan and Knowles 1972, Montesano et al. 1973). Dewaide (1971) has reviewed a wide range of studies concerning the properties of drug-metabolizing enzymes in fish. More recently there have been reports of mixed function oxidase activity in many extrahepatic tissues in *Raja erinecea* (Bend et al. 1973) and *Stenotomus bersicolor* (Stegeman et al. in press). The great gap in the knowledge of the disposition of foreign compounds in fish is caused by the lack of *in vivo* studies that are needed to assess the functional significance of the *in vitro* work that has demonstrated the presence of these enzymes.

Several reports document the occurrence of biotransformation reactions in fish *in vivo*. Huang and Collins (1962), in a study of the disposition of injected *p*-nitrobenzoic acid in flounder, dogfish, and goosefish, demonstrated the presence of several polar conjugates of this compound in the urine collected during the experiment, even though an adequate concentration gradient was maintained for outward gill diffusion. These conjugates were thought to be derivatives of glycine, glucuronic acid, and acetic acid. Lotlikar et al. (1967) reported that 2-acetoaminofluorene is hydroxylated by rainbow trout *in vivo*, and Hunn et al. (1968) have noted the presence of the N-acetyl-derivative of the fish anesthetic, tricainemethanesulfonate, in blood of trout exposed to this agent. Gutenmann and Lisk (1965) noted the *in vivo* conversion of 4-(2,4-dichlorophenoxybutyric acid [4-(2,4-DB)] to 2,4-dichlorophenoxyacetic acid (2,4-D) by bluegills. In an evaluation of endosulfan (Thiodan) as a fish-control agent, Schoettger (1970) reported the occurrence of a conjugated metabolite in the bile of white suckers, but this compound was not characterized. Rotenone, an insecticide and fish-control agent, can apparently be metabolized by carp *in vivo* (Fukami et al. 1969). In a study of the dynamics of 2,4-D in channel catfish and bluegills, Shultz (1973) reported the occurrence of as many as six metabolites of this compound in fish muscle.

A study of ^{14}C-2,4-D in bluegills (Macek et al. 1975) showed not only that metabolism is apparently important in the elimination of some chemicals from fish but also that some chemicals may induce systems responsible for their own metabolism. Some of the chemicals studied by this group were accumulated by bluegills so that tissue levels increased for 1-2 weeks or even longer. Others, however, showed this accumulation for only a few days followed by an almost complete loss of tissue ^{14}C despite continued exposure, suggesting that some process responsible for rapid elimination may have been initiated.

In vivo studies using marine fish have indicated that the species studied were able to hydroxylate several components of petroleum oil and that the bile was a major storage site of the polar metabolites (Lee et al. 1972). The information gained is of great significance since the elimination of these highly lipid soluble compounds from the species studied had a component which was dependent upon biotransformation rather than solely outward diffusion of the unchanged chemical. Another report indicated that rainbow trout can hydroxylate 3,4-benzpyrene, a carcinogenic constituent of crude oil (Pederson et al. 1974).

One of the most interesting problems in this area concerns the effects of inducers on microsomal enzyme activity in various species of fish. One of the earliest studies with DDT (Buhler 1966) indicated that several hepatic microsomal enzymes could be induced in trout, but subsequent reports demonstrated great variability in response to both DDT and phenylbutazone (Bend et al. 1973, Addison et al. 1977). Dewaide (1971) reviewed a wide range of work concerning the properties of microsomal enzymes in fish and suggested that the levels of activity and response of microsomal enzymes to inducers could be important in determining the susceptibility of fish to toxic substances in the environment. The application of concepts gleaned from studies in mammals demonstrates that inducers of the 3-methylcholanthrene (3-MC) type can induce arylhydrocarbon hydroxylase (AHH) activity in several marine species (Payne and Penrose 1975, Bend et al. 1973). In fact, the level of AHH activity in trout has been closely correlated with the level of oil pollution in some bodies of water (Payne 1975).

Goals of the Research

For the present project we selected for investigation several questions whose answers will increase knowledge of the ability of fish to cause the biotransformation of xenobiotics. Studies of these biotransformations *in vitro* have focused on the liver because of its central role in such reactions in the intact fish. Published work on this subject has utilized homogenization and fractionation of liver by the same methods used for mammalian liver without characterizing the fractions obtained from fish liver. We will therefore begin with the characterization of the subcellular fractions of rainbow trout liver and proceed with validation of various enzyme assays in the appropriate fraction(s). These assays are utilized for characterization of xenobiotic metabolizing activity. Subsequently, the xenobiotic metabolizing ability [microsomal monooxygenase, cytochrome(s) P-450] of the livers of normal and induced trout will be characterized.

Biotransformation also will be evaluated *in vivo* by characterization of the metabolites found in bile and tissues of the organic pollutants listed earlier after exposure of fish to the ^{14}C-labeled chemicals. The effect of inducers and inhibitors on the production of such metabolites also will be examined.

ACCUMULATION AND ELIMINATION OF XENOBIOTICS BY FISH

The accumulation of water-borne chemicals in fish is dependent on a variety of processes. Although many studies have been carried out on the uptake and bioaccumulation of organic chemicals in fish, little has been done in studying a series of related chemicals in order to correlate their structural features with the processes which govern the biological disposition of foreign chemicals. Fromm and Hunter (1969) reported that dieldrin was transferred across perfused rainbow trout gills when blood or blood plasma was used as the perfusing solution but not when a non-lipid solution was used. They suggested that the dieldrin was more soluble in blood (containing lipoprotein) than in the exposure water and therefore crossed the gill barrier into blood, thereby entering tissues where even greater solubility of dieldrin in tissue lipids exists. Hamelink et al. (1971) expanded this idea and suggested that in general organochlorine compounds of low water solubility are accumulated on the basis of exchanges dependent on increasing lipid character.

More recently, Neely et al. (1974) related bioaccumulation of a given compound to its octanol-water partition using the well-known Hansch coefficient utilized by the pharmaceutical industry. These workers reported a linear relationship between the log of the partition coefficient and the log of the biocencentration factor for many compounds. This method, however, and any other method which predicts bioaccumulation solely on the water solubility of the compound, ignores the possibility of metabolism of the compound in fish. These investigators also have proposed an accelerated method for predicting bioconcentration by use of the rate constants for uptake and washout of a chemical by fish (Branson et al. 1975). Their method, however, treats dilution by growth as elimination and may therefore be misleading.

Relatively little information is available in the reviewed literature on the uptake and disposition of coal constituents by freshwater fish. Apparently, the most water soluble (water-extractable) constituents of coal and petroleum are similar. Among the 20 chemicals found in an aqueous coal leachate were naphthalene, two methylnaphthalenes, and two dimethylnaphthalenes (Carlson and Caple 1979). Naphthalene and methylnaphthalenes are also among the substituents of petroleum and petroleum products which are most readily extracted into water (Boylan and Tripp 1971, Anderson and Neff 1975, Larson and Weston 1976). Furthermore, naphthalene and methylnaphthalenes are the organics appearing in the Gulf Killifish (*Fundulus similus*) at the highest levels following exposure to the water-soluble fraction of No. 2 fuel oil (Neff et al. 1976). The several reported studies of the uptake of naphthalene and related chemicals by marine species of fish are therefore of interest. Lee et al. (1972) studied the uptake, metabolism, disposition, and elimination of ^{14}C-naphthalene and ^{3}H-3, 4-benzopyrene by mudsucker (*Gillichtys mirabili*), tidepool sculpins (*Oligocottus maculosus*), and sand dab (*Citharichthys stigmaeus*). During exposure to either of these chemicals, fish of all three species accumulated ^{14}C or ^{3}H to levels very much higher than the aqueous exposure level during short term exposure (2 h or less). When the fish were placed in fresh seawater following exposure to the labeled chemical, the ^{14}C accumulated from ^{14}C-naphthalene was released very rapidly, while the ^{3}H from ^{3}H-3,4-benzopyrene was released much more slowly. Gall bladder bile, urine, and tissue extracts from these fish were examined for the presence of biotransformation products by thin-layer chromatography. The radioactive peaks were tentatively identified by comparison to published R_f values. For both chemicals the major product appeared to be a dihydrodiol.

In order to predict accurately the bioaccumulation of a chemical in fish the ability to incorporate a metabolism factor into the expression of the phenomena of uptake and elimination by the whole organism is important. The ability of rainbow trout to metabolize the lampricide TFM by formation of TFM-glucuronide is so important that it apparently explains the difference in toxicity of TFM to rainbow trout and to lampreys, which cannot form TFM-glucuronide (Lech and Statham 1975).

In this study we will examine the uptake and elimination of the chemicals listed under organic pollutants. In addition the effects of inducers or inhibitors of biotransformation on tissue levels, on rates or elimination, and on the form of the pollutant present will be examined. In particular, we will attempt to evaluate the role of metabolism in the accumulation and elimination of these chemicals.

Previous studies in this laboratory have shown that certain foreign chemical and/or their metabolites appear in fish bile at concentrations many times higher than the concentrations in the water (Statham et al. 1976). We have suggested that bile sampling might therefore serve as a monitoring method for selected environmental polutants. With this in mind, the biliary forms of the various chemicals under study will be characterized.

METHODOLOGY

Fish

Rainbow trout (*Salmo gairdneri*) were obtained from the Kettle Moraine Springs Trout Hatchery, Adell, Wisconsin, and bluegill sunfish (*Lepomis macrochirus*) and carp (*Cyprinus carpio*) were supplied by the U.S. Department of the Interior, Fish and Wildlife Service, Lake Mills National Fish Hatchery, Lake Mills, Wisconsin, and Fish Control Laboratory, LaCrosse, Wisconsin. These fish were maintained in flowing charcoal-filtered tap water at 12°C (the temperature at which the experiments were performed) for at least 1 week prior to use and were fed commercial trout pellets 3 times/week. A cycle of 12 h light, 12 h dark was used. Exposures of saltwater sheepshead (*Archosargus probatocephalus*) were performed at the National Institutes of Environmental Health Sciences Laboratory at Marineland, Florida, at 15°C on the day following capture.

Pretreatment of Fish for Studies of Induction of Hepatic Xenobiotic Metabolizing Activity

β-Naphthoflavone, 3-methylcholanthrene, 2,3-benzanthracene, Aroclor 1254, Aroclor 1242, and Firemaster BP6 were administered to trout by intraperitoneal injection as solutions in corn oil (1 ml/kg). Doses of these compounds varied from 0 to 275 mg/kg. Phenobarbital (65 mg/kg) was administered intraperitoneally as an aqueous solution (1 ml/kg). Control fish received either corn oil or water alone. Careful injection resulted in no observable leakage of compounds from the injection site. After injection fish were kept in 50 liter tanks (5-7 fish/tank) until sacrifice.

Exposures of Fish to ^{14}C-labeled Chemicals

Static Exposures--

In order to study the uptake, elimination and metabolism of the ^{14}C-labeled chemicals by fish, some experiments were conducted using relatively short exposure times. When such experiments required exposure to the ^{14}C-labeled chemical for 36 h or less, the fish were exposed to the chemical in a non-flowing aqueous system. When the exposures were performed for the purpose of studying biotransformation, the fish were sacrificed at the end of the exposure period and treated as described in later sections on Tissue Treatment and Metabolite Studies. When exposures were performed for the purpose of studying uptake and elimination, fish were sacrificed at various times during the exposure period and during a subsequent elimination period in fresh-flowing water and treated as described under Tissue Treatment.

Continuous Flow Exposures--

For uptake and elimination studies, rainbow trout (3-6 g), carp (2-6 g), or bluegill sunfish (0.7-1.4 g) were maintained in aqueous ^{14}C-labeled naphthalene, 2-methylnaphthalene, or 1,2,4-trichlorobenzene for 4-5 weeks. The concentration of these chemicals in the exposure water averaged 0.013 to 0.023 mg/liter, and the individual values are included with the results. The continuous-flow delivery system was similar to the system of Mount and Brungs (1967) as modified by DeFoe (1975). A solution of the ^{14}C-labeled chemical in acetone was added to the water at 3 μliter/liter. The water flow was approximately 8 liters/h. Because of the high volatility of the chemicals being studied, the water was aerated in the distributing system before the ^{14}C-labeled chemical was added. The exposure was initiated by the addition of the fish to 95 liters of water containing the appropriate chemical. Groups of five fish were removed at intervals for the determination of tissue levels of ^{14}C. After approximately 4 weeks of exposure to the ^{14}C-labeled chemical, the remaining fish were transferred to fresh-flowing water to monitor the elimination of that chemical. Again, groups of five fish were removed at suitable intervals to determine tissue ^{14}C levels. Water levels of each chemical were monitored by counting the ^{14}C present in aliquots of the water during the exposure and elimination periods. The fish were fed twice per week, and sampling was usually done at least 2 days after feeding.

Effect of Induction of Liver Xenobiotic Metabolizing Enzymes on Disposition and Metabolism of Chemicals *in Vivo*

Rainbow trout (80-100 g) were treated with corn oil or 2,3-benzanthracene in corn oil as described earlier. Both groups were placed in tanks for 48 h after which the trout were exposed to ^{14}C-2-methylnaphthalene (0.05 mg/liter) in 50-liter tanks for 6 h. The fish were returned to fresh-flowing water and sampled at various intervals for the determination of ^{14}C-labeled material in blood, muscle, liver, and bile.

Groups of eight rainbow trout (70-120 g) were treated with corn oil or a solution of BNF in corn oil as described above. After 48 h the groups of control fish (corn-oil injected) and induced fish (BNF-injected) were placed in tanks containing ^{14}C-labeled naphthalene, 2-methylnaphthalene, or 1,2,4-trichlorobenzene in 50 liters of water. The fish were sacrificed after 24 h exposure to the ^{14}C-labeled chemical (72 h after the injections). Samples of bile, blood, liver, and muscle were taken. These samples were used for ^{14}C measurements, for determining the percentage of ^{14}C present as metabolites (polar material), or both.

Studies also were conducted on the effects of the microsomal inhibitor piperonyl butoxide on the biotransformation of pentachloroanisole and di-2-ethylhexylphthalate by rainbow trout *in vivo*. In these experiments the trout were exposed to 1 mg/liter piperonyl butoxide for 24 h, followed by coexposure to piperonyl butoxide plus the ^{14}C-labeled chemical for 24 h.

Preparation of Subcellular Fractions of Liver

The procedure for the preparation of subcellular fractions of fish liver used in the initial studies is summarized in Figure 1. Fish were sacrificed by cervical dislocation. The livers were removed, minced, and subjected to the homogenization and fractionation procedures listed in Figure 1. A 1 g sample of fish liver was assumed to have a volume of 1 ml and the livers were homogenized in four volumes of 0.25 *M* sucrose. All operations were carried out at 4°C. Each particulate fraction was suspended in 0.25 *M* sucrose in a volume equal to 16.7% of the starting homogenate and was repelleted at the same gravitational (*g*) force. The second supernatant was combined with the original supernatant obtained for the respective fraction and subjected to the next higher *g* force to obtain the subsequent fraction. All particulate fractions were suspended finally in a volume of 0.25 *M* sucrose equal to that of the starting homogenate. To give a more homogenous suspension, all membranous fractions (including starting homogenate fractions) were sonified for 3 x 10 sec periods separated by 30 sec periods with a Branson Sonifier Cell Disruptor Model W140 D (20,000 Hz,

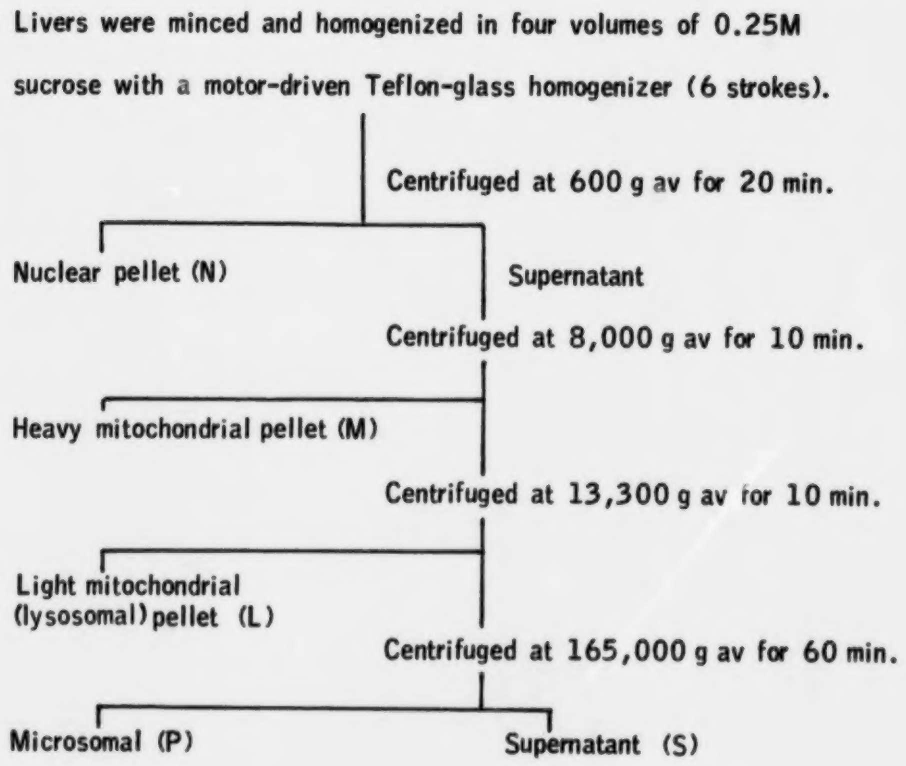
Livers were minced and homogenized in four volumes of 0.25M sucrose with a motor-driven Teflon-glass homogenizer (6 strokes).
Centrifuged at 600 g av for 20 min.
Nuclear pellet (N)
Supernatant
Centrifuged at 8,000 g av for 10 min.
Heavy mitochondrial pellet (M)
Centrifuged at 13,300 g av for 10 min.
Light mitochondrial (lysosomal) pellet (L)
Centrifuged at 165,000 g av for 60 min.
Microsomal (P)
Supernatant (S)

Figure 1. Scheme for fractionation of rainbow trout liver.

400 watts), amplitude setting of 6, fitted with a microtip. Between each sonification period, a 30 sec period of cooling at 4°C was introduced to prevent temperature increases.

A slightly different protocol was used when the only subcellular fraction required was the microsomal fraction. In this case the livers were minced and washed 3 times with ice-cold 0.154 *M* KCl to remove adhering hemoglobin. The minced livers were homogenized for six complete strokes in four volumes of 0.25 *M* sucrose using a motor-driven Potter-Elvejhem glass-teflon homogenizer. The homogenate was centrifuged at 8,500 x *g* (r_{av} = 8.3 cm) for 20 min using a Sorvall-type-24 rotor and RC-5 centrifuge. The resulting supernatant was centrifuged at 165,000 *g* (r_{av} = 5.7 cm) for 60 min using a Beckman type 65 rotor and Model L5-65 ultra-centrifuge. The microsomal pellet obtained was resuspended in 0.154 *M* KCl and the microsomes residimented at 165,000 x *g* for 60 min. The washed microsomal pellet was resuspended in 0.25 *M* sucrose to give a final concentration equivalent to 1 g wet weight liver/ml. All operations were performed at 0-4°C, and the microsomes were utilized on the day of preparation.

A different protocol also was used to prepare trout liver subcellular fractions for studying metabolism of ^{14}C-di-2-ethylhexylphthalate so that results could be compared to those reported for another species (Stalling et al. 1973). The livers were quickly removed and placed in ice-cold 0.154 *M* KCl. The livers were weighed, minced, and rinsed with ice-cold 0.154 *M* KCl. A 20% homogenate of the livers in 0.154 *M* KCl was then made by five up-and-down cycles of a motor-driven homogenizer of the Potter-Elvejhem type having a glass vessel and a Teflon pestle.

Subcellular fractions were prepared by differential centrifugation at 4°C. The liver homogenate was first centrifuged for 10 min at 2,000 g in a Sorvall model RC-5 refrigerated centrifuge. The pellet from this centrifugation generally was not utilized, but the supernatant fraction was used as prepared or was further fractionated in a Beckman model L-2 ultracentrifuge. The pellet resulting from a 20-min centrifugation of the 2,000 *g* supernatant fraction at 10,000 *g* was resuspended with use of the tissue homogenizer in a volume of 0.154 *M* KCl equal to or less than the volume of the supernatant fluid removed. This resuspended fraction was referred to as the mitochondrial fraction. The 10,000 x *g* supernatant fraction was centrifuged at 100,000 x *g* for 1 h. The 100,000 x *g* pellet was resuspended in 0.154 *M* KCl as described for the 10,000 x *g* pellet, and was designated the microsomal fraction.

In studies in which serum was used, blood was obtained from the caudal vein of rainbow trout and allowed to clot. The remaining liquid was centrifuged at 1,500 x *g* for 10 min, and the supernatant fluid was diluted 5- or 10-fold with 10 m*M* phosphate buffer, pH 7.2.

Enzyme Assays and Spectral Measurements

Succinic dehydrogenase activity was measured using a reaction mixture containing 10 m*M* phosphate (pH 7.4), 10 mg bovine plasma albumin, 1 m*M* KCN, and 1 mg horse heart cytochrome C (Green et al. 1955) in a final volume of 0.9 ml. After recording the baseline at room temperature, the reaction was initiated by adding 0.1 ml of 50 m*M* sodium succinate, and the change in absorbance was measured at 550 nm with a 1 cm path in a spectrophotometer (Gilford) with a recorder.

Acid phosphatase was assayed using *p*-nitrophenyl phosphate as substrate (Gianetto and deDuve 1955). The β-glucuronidase activity was measured with phenolphthalein glucuronide as substrate (Gianetto and deDuve 1955). Acid phosphatase, β-glucuronidase and all enzyme assays described below were carried out at 25°C. This temperature is considered optimal for enzymatic activities of fish liver (Dewaide 1971) rather than 37°C, which is a usual temperature for assaying many enzymatic activities of mammalian preparations. The acid phosphatase and β-glucuronidase assays were stopped, in the first case by the addition of 1.0 ml of 2.0 *M* glycine (pH 10.7) and in the second by the addition of 1.5 ml of 0.13 *M* glycine, 60 m*M* NaCl, and 80 m*M* Na_2CO_3 (pH 10.7). The reaction tubes were centrifuged at approximately 30,000 x *g* for 10 min before absorbance was read at 420 nm.

Cathepsin D activity (Gianetto and deDuve 1955) was assayed using a final concentration of 0.5% hemoglobin as substrate. After the reaction was stopped with 1 ml of 5% trichloracetic acid (TCA), the samples were centrifuged at approximately 30,000 x *g* for 10 min and the adsorbance read at 280 nm.

Alkaine phosphatase was assayed by incubating the enzyme preparation in a final volume of 0.5 ml containing 0.1 *M* ethanolamine (pH 9.5), 1.5 m*M* sodium floride, and 5 m*M* *p*-nitrophenyl phosphate at 25°C for 30 min. After the reaction was terminated with 1 ml of 2 *M* glycine (pH 10.7), the samples were centrifuged at approximately 30,000 x *g* for 10 min before the adsorbance was read at 420 nm.

Lactic dehydrogenase activity was assayed with a reaction mixture containing 50 m*M* TES [sodium *N*-tris (hydroxylmethyl) methyl-2-aminoethane sulfonic acid], 1 m*M* dithiothreitol, and 0.2 m*M* NADH. After the baseline was recorded, the reaction was initiated with 0.1 ml of 0.1 *M* pyruvate and the change in adsorbance was measured at 340 nm.

Uridine diphosphoglucuronic acid (UDPGA)-glucuronyl transferase (indicated subsequently as glucuronyl transferase) was assayed at 25°C with a reaction mixture containing 100 m*M* sodium phosphate (pH 7.0), 0.2 m*M* $MgCl_2$, 5 m*M* saccharo-1,4-lactone, 0.5 m*M* UDPGA, and 0.5 m*M* ^{14}C-3-trifluoromethyl-4-nitrophenol containing approximately 3.0×15^5 counts/min (CPM) in a final volume of 1.0 ml. The reaction was stopped after 10 min with 0.2 ml of 10% TCA, which was followed by the addition of 1.0 ml water to each tube. Extraction of unreacted, labeled substrate was done with 4 ml of benzene:ether (1:1 v:v) using a 10 min shaking time with a mechanical shaker after which the upper phase was removed by aspiration. This procedure was repeated twice. An aliquot (0.5 ml) of the lower aqueous phase was counted in 15 ml of ACS scintillation cocktail (Amersham/Searle). A reaction tube without added UDPGA was assayed for each cellular function, and the radioactivity found under these assay conditions was subtracted from that found in the presence of UDPGA.

The 5'-nucleotidase assay was carried out at 25°C with a reaction mixture containing 50 m*M* TES (pH 7.5), 1 m*M* ethylendiamine tetracetic acid (EDTA), 5 m*M* $MgCl_2$, and 0.1 m*M* ^3H 5'-adenosine monophosphate (AMP) containing approximately 50,000 CPM in a final volume of 250 μliter. After the incubation period (20 min), the reaction was terminated by boiling for 1 min. The ^3H-adenosine was separated from the labeled substrate on a small 4.5 cm column of DEAE-Sephadex A-25, contained within a Pasteur pipette. A similar "pencil" column technique has been described for the separation of labeled adenosine from cyclic AMP in the assay of cyclic nucleotide phosphodiesterase (Huang and Kemp 1971). The ^3H 5'-AMP, eluted with 3.0 ml 50 m*M* Tris HCl (pH 7.5), was counted in a Packard liquid scintillation spectrometer (Model 3310) with 10 ml of a premixed scintillation cocktail (ScintiVerse, Fisher Scientific Company). An aliquot of the assay mixture containing ^3H 5'-AMP was counted in 3.0 ml 50 m*M* Tris HCl buffer under the same conditions as the assay samples and no quench correction was required.

Rotenone-insensitive NADH or NADPH-cytochrome C reductase was assayed in the presence of 1.5 μ*M* rotenone and the reduction of cytochrome C at 550 nm was followed (Sottocasa et al. 1967). Rotenone for the enzyme assay was prepared by the drop-wise addition of 200 μliter of 0.225 *M* rotenone in dioxane in 10 ml of 10% albumin; 0.1 ml of this mixture was used in the assay to give a final rotenone concentration of 1.5 μ*M*.

Glucose-6-phosphatase activity was assayed at pH 6.0 by measuring the inorganic phosphate released from glucose-6-phosphate in a reaction mixture containing KF and EDTA to minimize interference in the assay by acid and alkaline phosphatase activities (Hubscher and West 1965). After the reaction was stopped with 10% TCA, the samples were centrifuged at approximately 30,000 x *g* for 10 min, and an aliquot of the clear supernatant was assayed for inorganic phosphate with ascorbic acid and ammonium molybdate (Ames 1966).

The benzopyrene hydroxylase assay, used in the present study, was based on the assay initially described by Hansen and Fouts (1972). The reaction mixture contained 0.1 *M* Tris-HCL (pH 7.4), 5 m*M* glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 1 m*M* NADP, and 5 μ*M* ^{14}C-benzo[a]pyrene containing approximately 10 CPM and enzyme in a final volume of 2.5 ml. The reaction stopped after 5 min with 1.0 ml of cold acetone and placed in an ice bath (4°C). Hexane (5.0 ml) was added to each sample and the mixture was shaken for 20 min. Then 2 *N* NaOH (2.5 ml) was added to each tube, and the shaking was continued for an additional 20 min. The samples were centrifuged at 2,500 rpm with an IEC centrifuge for 10 min, the upper (hexane) layer was removed by aspiration, and a 1.0 ml aliquot of the aqueous layer was counted in a liquid scintillation spectrometer with 15 ml ACS scintillation cocktail in the presence of 100 μliter of glacial acetic acid to minimize chemiluminescense.

Ethylmorphine-*N*-demethylation was determined by measurement of liberated HCHO by a modified method of Anders and Mannering (1966).

7-Ethoxycoumarin-*O*-deethylation was measured by the direct fluorimetric procedure of Ullrich and Weber (1972). Final quantities of reactants in the cuvette were 100 nmole of NADPH, 3.2 mole of glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 5-450 nmole of ethoxycoumarin, 0.1-0.3 mg of microsomal protein, and 66 m*M* Tris-HCl buffer (pH 7.4) in a final volume of 1 ml.

7-Ethoxyresorufin-*O*-deethylation was determined by the method of Burke and Mayer (1974). Final quantities of reactants in the cuvette were 500 nmol NADPH, 0.1-0.5 mg microsomal protein, 15-600 pmol ethoxyresorufin, and 66 m*M* Tris-HCl buffer (pH 7.4) to 1 ml total volume.

Temperature optima for these monooxygenase assays were 25°C for arylhydrocarbon hydroxylase and ethylmorphine-*N*-demethylase and 29-30°C for ethoxycoumarin- and ethoxyresorufin-*O*-deethylases.

Protein was determined by the method of Ross and Schatz (1973) using crystalline bovine plasma albumin as the standard.

Spectral Measurements--

All measurements were made using either an Aminco DW2 UV/VIS Spectrophotometer or a Cary 219 Spectrophotometer. The λ_{max} for the CO complex of $Na_2S_2O_4$-reduced hemoprotein(s) P-450 were determined at a protein concentration of 1-2 mg/ml. The spectrophotometer was calibrated using a holmium oxide filter before and after use. An extinction coefficient of 100 mM^{-1} cm^{-1} for the difference in absorbance between 450 and 510 nm of the carboxyferrocytochrome P-450 minus ferricytochrome P-450 difference spectrum was utilized (Estabrook et al. 1972). This method involves reducing the contents of the sample cuvette with $Na_2S_2O_4$ and gassing both cuvettes with CO, hence eliminating spectral interference due to haemoglobin.

Type I and type II oxidized binding spectra of various compounds with the trout hepatic microsomes were obtained as described by Schenkman et al. (1967). The substrates were dissolved in *N,N*-dimethylformamide and added as μliter quantities to the sample cuvettes. Equivalent quantities of dimethylformamide were added to the reference cuvette. Dimethylformamide did not elicit any binding spectra of its own at the concentrations employed in this study.

Ethylisocyanide-ferrocytochrome P-450 difference spectra were obtained at pH 7.4 as described by Imai and Sato (1966).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at room temperature using a modified system of Laemmli and Favre (1973) as described by Dent et al. (1978). A 15-cm-long 7.5% acrylamide resolving gel was utilized. Staining for protein was with Coomassie brilliant blue R-250 and for peroxidase activity the precedure of Thomas et al. (1976) was used.

DEHP Metabolism *in Vitro*--

The *in vitro* metabolism of DEHP was assessed by incubation of the various tissue fractions with ^{14}C-DEHP and determination of the amount of

DEHP remaining and of metabolites formed during the incubation. The incubation solution consisted of 0.4 ml of tissue preparation in 0.154 *M* KCl, 0.4 ml of 10 m*M* phosphate (pH 7.2), 0.1 ml of 10 m*M* MgCl$_2$, and either 0.1 ml of 20 m*M* NADPH (pH 7.2) or 0.1 ml of water/ml of incubation medium. Total volume was 1.0 or 2.0 ml. The addition of compounds of low water-solubility, DEHP, 2,4-DBE, and piperonyl butoxide (PBO), was accomplished by the addition of small volumes (5-40 µliter) of solutions of these materials in methanol, which was evaporated off before addition of the aqueous components. ^{14}C-DEHP was present at 5 µ*M*, ^{14}C-2,4-DBE at 10 µ*M*, and PBO at 2 m*M*. A slightly different method for the addition of PBO was utilized because of its low solubility in water. A premix of the ionic constituents of the incubation medium was shaken with PBO followed by centrifugation and decanting of the aqueous layer. This premix with PBO was added to ^{14}C-DEHP. The concentration of PBO in the premix was determind (Bhavnagary and Ahmed 1973) and found to result in a final concentration of 9 x 10^{-5} *M* PBO.

Incubations were initiated by the addition of the appropriate tissue fraction to a chilled tube containing the other required materials which was placed in a Dubnoff Metabolic Shaker at 22°C. Incubations were terminated by the addition of 0.2 ml of 10 *N* HCl. Extraction of the acidified incubation medium three times with 1.0 ml of diethyl ether removed over 98% of the radioactivity. The pooled ether extracts from each tube were reduced in volume to approximately 0.3 ml to facilitate further analysis. The extent and nature of the metabolism occurring during these incubations were evaluated by thin layer chromatography (TLC) of the extracts on plates coated with silica gel containing a UV indicator which served to locate the phthalate standards which were run along with the incubation extracts. The solvent systems used were CHCl$_3$:MeOH:HOAc in the ratios 143:7:2 and 5:1:1 (v:v:v) for the DEHP studies, and hexane:acetone (1:1) for 2,4-DBE. The developed plates were monitored using a Packard Model 7201 Radiochromatogram Scanner to locate the radioactive peaks which were quantified by scraping gel segments and counting them in a scintillation counting system (Searle Analytic Company Model 300). The percentage of total ^{14}C present in each metabolite peak was calculated and converted to nanomoles based on initial nanomoles of DEHP and 2,4-DBE.

Tissue Treatment for ^{14}C Determinations

Aliquots of bile and blood were added to 15 ml of ACS scintillation mixture for ^{14}C counting. Similarly, aliquots (1 or 2 ml) of the various exposure solutions were monitored for ^{14}C; all exposure levels of ^{14}C-labeled chemicals specified in the results are based on these measurements. Weighed portions of solid samples were dissolved in NCS tissue solubilizer at 48°C for 24 h before addition of the ACS scintillation mixture. On some occasions entire small fish or carcasses were dissolved and aliquots of the resulting solution were used for ^{14}C determinations.

After addition of the scintillation mixture each vial received 40 µliter of glacial acetic acid followed by 24 h of heating at 48°C and 24 h of cooling before counting to reduce chemiluminescence. Radioactivity counting was done with a Searle Analytic Company Isocap/300 Liquid Scintillation System. A computer program was used to convert the radioactivity data to the amount of parent ^{14}C-labeled chemical plus metabolites present in each tissue expressed as μg/g.

Metabolite Studies

Bile was examined for the presence of metabolites of the ^{14}C-labeled chemicals by TLC or by solvent partitioning. After TLC, 1-cm segments of the silica gel from origin to solvent front were scraped and subjected to radioactivity counting. The locations of the radioactivity were compared to those of appropriate standards. Aliquots of bile were partitioned between 2 ml of hexane and 2 ml of dilute phosphate or 2-amino-2-(hydroxymethyl)-1,4-propanediol buffers (pH 7.4 or 11.0) followed by measurement of the radioactivity present in each phase. After partitioning at pH 7.4 only conjugated or acidic metabolites appeared in the aqueous phase, while at pH 11 the phenolic metabolites also appeared in the aqueous phase.

Muscle and liver tissues were placed in cold acetone at the time of dissection. They were homogenized using a Waring blender in a chilled homogenization vessel. The tissues were extracted at least twice with acetone, once or twice with hexane, and usually at least once with ethanol or methanol. In the case of muscle this final alcohol extraction included an additional homogenization. Extractions of radioactivity were at least 95% complete as evaluated by digestion and radioactivity measurements on aliquots of the extraction residues. Aliquots of these extracts were examined by TLC and hexane-water partitioning or both to evaluate the presence of metabolites.

To assist in the identification of possible glucuronide conjugates of biotransformation products, hydrolysis based on catalysis by β-glucuronidase was utilized.

Aliquots of solutions of the metabolites were evaporated to dryness and the residue was dissolved in 0.05 *M* phosphate buffer (pH 6.8). β-glucuronidase solution (1 Sigma unit/μliter) was added to the metabolite solution to give a final concentration of 200 units/ml, and the final solution was incubated at room temperature. In some cases, duplicate aliquots were utilized to check whether saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase (Levvy 1952), inhibited the hydrolysis. When the hydrolysis was done for analytical purposes, an aliquot of the incubation solution was applied directly to a TLC plate. When the hydrolysis was done for preparative purposes, the incubation solution was extracted several times with diethyl ether after acidification. In this report the term "hydrolyzed metabolite" refers to the results of *in vitro* incubation with β-glucuronidase rather than to any *in vivo* metabolic reaction.

Thin-Layer Chromatography

Aliquots of bile and of various tissue extracts were subjected to TLC on silica gel plates using various solvents, depending upon the compounds of interest. The various samples were co-chromatographed with appropriate standards. After chromatography the standards were located by use of a hand-held shortwave UV light while the radioactivity was located by scraping a 1 cm segment of the silica gel from origin to solvent front and scintillation counting of the individual segments.

Materials

NADP$^+$, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 7-hydroxycoumarin (umbelliferone), saccharo-1,4-lactone, -glucuronidase (bacterial type II), phenobarbital, and UDPGA were obtained from Sigma Chemical Company (St. Louis, Missouri).

7-Ethoxycoumarin was synthesized by the method of Ullrich and Weber (1972) and 7-ethoxyresorufin (7-ethoxyphenoxazone) was synthesized by Dr. S. R. Challend (Wellcome Research Labs, Beckenham, Kent, U.K.) from resorufin (7-hydroxyphenoxazone, Eastman Organic Chemicals, Rochester, New York).

Aroclors 1254 and 1242 were generous gifts from Monsanto Chemical Company while metyrapone (Metopirone) was kindly donated by the Ciba-Geigy Corporation (Sumitt, New Jersey). Dr. D. Rickert (Chemical Industry Institute of Toxicology, Raleigh, North Carolina) generously donated Firemaster BP6.

β-Naphthoflavone (5,6-benzoflavone), and α-naphthoflavone were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin).

5'-AMP was obtained from PL Biochemicals, Milwaukee, Wisconsin, and Fraction V bovine plasma albumin from Rheis Chemical Company, Kankakee, Illinois.

Ethyl isocyanide was kindly donated by Dr. R. Philpot (National Institute of Environmental Health Sciences, North Carolina).

All other chemical reagents and solvents used in the enzyme assays were of the highest commercial quality available.

8-^{14}C-2-Methylnaphthalene (specific activity, 8.0 mCi/mmol) was supplied by California Bionuclear Corp. (Sun Valley, California).

1-^{14}C-Naphthalene, 7,10-^{14}C-benzo[a]pyrene (51 mCi/mmol), and 2-^{14}C-phenobarbital (14 mCi/mmol) were purchased from Amersham/Searle, Des Plaines, Illinois.

3-Trifluoromethyl-4-nitrophenol was obtained from Mr. John Howell, U.S. Department of the Interior, Hammond Bay, Michigan. Radioactive 3-trifluoromethyl-4-nitrophenol (^{14}C ring uniformly-labeled, specific activity, SA 3.7 mCi/mol) was obtained from the Mallinckrodt Chemical Company, St. Louis, Missouri.

8-^{3}H 5'-AMP (10 Ci/mmol) was purchased from New England Nuclear, Boston, Massachusetts. ^{14}C-(carboxyl label) phthalic anhydride (17.6 mCi/mmol) was purchased from ICN Pharmaceuticals Irvine, California. Di-2-ethylhexyl (ring ^{14}C) phthalate (10.52 mCi/mmol), ^{14}C-(ring-UL)-

pentachlorophenol (10.04 mCi/mmol) ^{14}C-(Ring-UL)-1,2,4-trichlorobenzene, and 1,2,4,-trichlorobenzene were purchased from Pathfinder Laboratories, St. Louis, Missouri.

Pentachlorophenol and naphthalene (certified grade) were obtained from FisherScientific Company, Itasca, Illinois, and 2-methylnaphthalene (gold label grade) from Aldrich Chemical Company, Milwaukee, Wisconsin.

Saffrole and tropitol were a gift from the McGormley-King Co. (Minneapolis, Minnesota); piperonyl alcohol, piperonal, and 1,3-benzodioxole were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin) and piperonyl butoxide was purchased from Pfaltz and Bauer (Flushing, New York).

MEHP was a gift from Dr. David E. Stalling, U.S. Department of the Interior, Columbia, Missouri.

Amberlite XAD-2 resin was obtained from Rohm and Haas Co., Philadelphia, Pennsylvania.

Tetrachloroquinone was donated by Dr. P. Gehring, Dow Chemical Co., Midland, Michigan, and tetrachlorohydroquinone was purchased from Eastman Chemical Co., Rochester, New York.

Glass-distilled solvents were purchased from Burdick and Jackson, Muskegon, Michigan. Precoated silica gel plates (Sil-G 25 and Sil-G 25 UV 254) were purchased from Brinkmann Instrument Company, Westbury, New York. NCS tissue solubilizer and ACS scintillation mixture were purchased from Amersham, Arlington Heights, Illnois.

^{14}C-labeled naphthalene and 2-methylnaphthalene were purified to greater than 99% purity by TLC on methanol-washed silica gel plates using CCl_4 or $CHCl_3$ as solvents. The ^{14}C-trichlorobenzene was $>$ 99% pure as received.

Pentachloroanisole and ^{14}C-pentachloroanisole were made by methylation, respectively of pentachlorophenol and ^{14}C-(Ring-UL)-pentachlorophenol with diazomethane. The pentachloroanisole was purified by recrystallization from hexane, and the ^{14}C-pentachloroanisole purified by TLC on silica gel plates with methylene chloride as solvent.

Di-2-ethylhexyl [carboxyl-^{14}C] phthalate was prepared by refluxing ^{14}C-labeled phthalic anhydride with excess 2-ethyl-1-hexanol in benzene with a trace of H_2SO_4 for 4 h. The solvent was removed by distillation, and the ^{14}C-DEHP was purified by TLC on silica gel in benzene:ethyl acetate (19:1, v:v).

SECTION 4

RESULTS

HEPATIC XENOBIOTIC METABOLIZING ACTIVITY IN RAINBOW TROUT

Fractionation and Subcellular Localization of Marker Enzymes in Rainbow Trout Liver

Several reports have appeared on the metabolism of xenobiotics by fish liver microsomes. These studies, however, have utilized protocols developed for studying liver microsomal metabolism in mammalian species. It is not known, therefore, whether these supposed "microsomal fractions" are indeed representative of the xenobiotic metabolizing enzymes of fish livers. Studies were therefore initiated to characterize the subcellular fractions obtained from a typical fractionation of rainbow trout liver homogenates (Statham et al. 1977).

Rainbow trout weighing 80-100 g were obtained and used for the preparation of liver subcellular fractions as described in Section 3.

Although the scheme described in Figure 1 was ultimately adapted to fractionate rainbow trout liver into subcellular fractions, preliminary experiments were carried out in an attempt to optimize conditions for a more definitive separation of "marker" enzymes characteristic of various subcellular components. Initially, a low speed spin at 120 x *g* for 5 min was included in the scheme described in Figure 1 to remove intact cells, but this resulted in variable distribution profiles, particularly for the plasma membrane markers, alkaline phosphatase and 5'-nucleotidase. Thus, in all the distribution profiles reported in this fractionation study, trout liver homogenates were centrifuged at 600 x *g* for 20 min to obtain the nuclear and cellular debris pellet (Figure 1).

Since the lysosomes are particularly important in protein and lipid degradation in mammalian liver, an attempt was made to optimize centrifugation conditions for a more definitive separation of the hydrolytic enzymes in trout liver homogenates. Lowering of the force of the mitochondrial spin or increasing that of the microsomal spin resulted in a lowered yield (percent recovery) of the mitochondrial or microsomal marker enzymes in their respective pellets without a significant increase of the relative specific activity (R.S.A.) of acid phosphatase in the light mitochondrial or lysosomal fraction.

Thus, in the standard fractionation scheme (Figure 1), the post mitochondrial fraction was centrifuged at 13,300 x *g* for 10 min to obtain the light mitochondrial or lysosomal pellet. The subcellular distribution of four hydrolytic or lysosomal marker enzymes in trout liver--fractionated according to the scheme described in Figure 1--were studied and the results reported in Table 1. These results show clearly that heterogeneity exists in percent recovery and relative specific activity of the four hydrolytic enzymes in the various subcellular fractions. β-Glucuronidase and N-acetyl-β-glucosaminidase yielded similar distribution profiles with most of the particulate activity in the nuclear and mitochondrial pellets but with little enrichment in the lysosomal fraction relative to the mitochondrial fraction. Almost 50% of Cathepsin D activity was recovered in the nuclear fraction. The R.S.A. of the enzyme was high and of similar value in both the nuclear and lysosomal pellets, indicating enrichment in each fraction. Acid phosphatase, a hydrolytic enzyme used most often to monitor lysosomal enrichment in mammalian liver fractionation schemes, yielded R.S.A. values that were similar for lysosomal and microsomal pellet, indicating a similar enrichment of this enzyme in both fractions. Since the major goal of this study was to explore the distribution profile of certain drug-metabolizing enzymes in trout liver, which are known to reside in the microsomal fraction of mammalian liver, further efforts in delineating a lysosomal fraction in trout liver were not made.

TABLE 1. DISTRIBUTION OF HYDROLYTIC ENZYMES OF RAINBOW TROUT LIVER[a]

	Nuclear pellet	Mito-chondrial pellet	Lysosomal pellet	Microsomal pellet	Supernatant 165,000xg_{av}
Acid phosphatase (6)					
Recovery, %	14.2	16.2	13.9	45.7	9.3
R.S.A.	0.8	1.3	1.8	2.1	0.2
β-Glucuronidase (4)					
Recovery, %	25.2	27.2	9.7	15.9	21.9
R.S.A.	1.7	2.3	1.3	0.8	0.5
N-Acetyl-β- Glucosaminidase (5)					
Recovery, %	26.7	25.6	10.6	11.5	25.5
R.S.A.	2.1	1.8	1.2	0.6	0.6
Cathepsin D (1)					
Recovery, %	47.2	14.4	12.8	18.3	7.2
R.S.A.	2.0	1.1	1.9	0.9	0.2

[a] Figures in parentheses indicate the number of experiments in which the enzymatic analyses were performed, and the values shown are averages of the indicated number of experiments. Relative specific activity (R.S.A.) = percent of total enzymatic activity:percent of total protein.

A full enzymatic characterization of subcellular fractions obtained by the scheme outlined in Figure 1 was undertaken, and the results are summarized in Table 2. Approximately 70% of the succinic dehydrogenase was located in the pellet obtained by centrifuging the post-nuclear supernatant for 8,000 x *g* for 10 min. The R.S.A. of succinic dehydrogenase of this fraction was 6.5, and the R.S.A. of the lysosomal fraction was 4.0, indicating some contamination of the lysosomal fraction by mitochondria.

TABLE 2. ENZYMATIC AND PROTEIN ANALYSES OF SUBCELLULAR FRACTIONS OF RAINBOW TROUT LIVER[a]

	Nuclear pellet	Mito-chondrial pellet	Lysosomal pellet	Microsomal pellet	Supernatant 165,000xg_{av}
Alkaline phosphatase (3)					
Recovery, %	36.2	4.0	3.0	36.5	20.2
R.S.A.	2.0	0.3	0.4	1.7	0.4
5'-Nucleotidase (3)					
Recovery, %	47.8	6.0	4.1	31.4	10.5
R.S.A.	2.4	0.6	0.6	1.4	0.3
Succinic dyhydrogenase (6)					
Recovery, %	16.2	67.6	16.2	0	0
R.S.A.	0.6	6.5	4.0	-	-
Acid phosphatase (6)					
Recovery, %	14.2	16.2	13.9	45.7	9.3
R.S.A.	0.8	1.3	1.8	2.1	0.2
Glucose-6-Phosphatase (6)					
Recovery, %	12.1	12.0	10.5	64.5	0.9
R.S.A.	0.6	1.1	1.6	2.9	-
Lactate dehydrogenase (3)					
Recovery, %	7.9	2.0	1.8	2.6	85.6
R.S.A.	0.4	0.2	0.3	0.1	2.2
Protein (8)					
Recovery, %	20.3	11.8	7.5	22.8	37.5

[a] Figures in parentheses indicate the number of experiments in which the enzymatic and protein analyses were performed, and the values shown are averages of the indicated number of experiments. Relative specific activity (R.S.A.) = percent of total enzymatic activity:percent of total protein.

Alkaline phosphatase and 5'-nucleotidase, markers of plasma membranes in mammalian liver (Salyam and Trams 1972), yielded almost identical distribution profiles. The percent recovery and R.S.A. values for these enzymes suggest that plasma membranes of trout liver, under the homogenization conditions described, were localized mainly in the nuclear and microsomal pellets (Table 2).

Of particular importance was the distribution of microsomal marker enzymes in subcellular fractions of trout liver prepared according to the scheme outlined in Figure 1. Glucose-6-phosphatase, a gluconeogenic enzyme found in the microsomal fraction of mammalian liver (Solyam and Trams 1972), was found to the extent of approximately 65% in the microsomal fraction of trout liver (Table 2, Table 3). This enzyme was definitely enriched in the microsomal fraction as indicated by the high R.S.A. of the fraction.

TABLE 3. DISTRIBUTION OF MICROSOMAL ENZYMES OF RAINBOW TROUT LIVER[a]

	Nuclear pellet	Mito-chondrial pellet	Lysosomal pellet	Microsomal pellet	Supernatant 165,000xg_{av}
Glucose-6-Phosphatase (6)					
Recovery, %	12.1	12.0	10.5	64.5	0.9
R.S.A.	0.6	1.1	1.6	2.9	-
Rotenone-insensitive cytochrome C reductase NADH (3)					
Recovery, %	14.1	16.0	12.4	57.2	0.2
R.S.A.	0.7	1.4	2.1	2.5	0.1
NADPH (2)					
Recovery, %	13.4	12.6	15.0	55.2	3.8
R.S.A.	0.7	1.2	3.0	2.2	0.1
Glucuronyl transferase (2)					
Recovery, %	17.8	9.1	8.6	63.5	0.1
R.S.A.	0.8	0.9	1.4	2.5	1.0
Benzopyrene hydroxylase (2)					
Recovery, %	15.9	5.2	5.2	63.3	10.5
R.S.A.	0.8	0.5	0.7	2.5	0.3

[a] Figures in parentheses indicate the number of experiments in which the enzymatic analyses were performed, and the values shown are averages of the indicated number of experiments. Relative specific activity (R.S.A.) = percent of total enzymatic activity:percent of total protein.

Microsomal contamination of the high-speed supernatant was low, but approximately 10-20% of the 5'-nucleotidase and alkaline phosphatase plasma membrane markers were found in the 165,000 x g supernatant (Table 2). Assay for lactate dehydrogenase indicated that approximately 85% of this enzyme was recovered in the high-speed supernatant, indicating minimal contamination of the membranous fractions by cytosolic material.

Since Table 2 is a composite of data accumulated over several experiments, the results from a single subcellular distribution in which all the enzymes and protein content were measured is presented in Figure 2. A comparison of the relative specific activities of the marker enzymes from

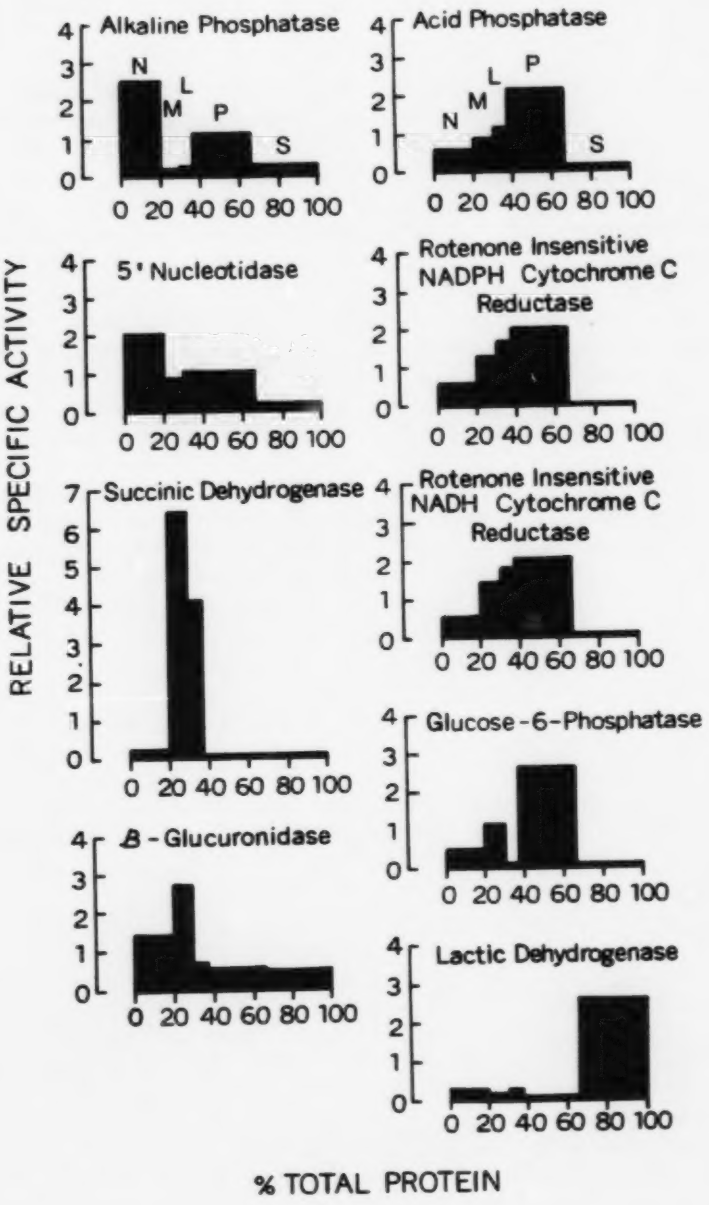

Figure 2. Distribution of marker enzymes from trout liver.

this individual fractionation with those presented in Table 2 indicates excellent agreement.

After the fractionation scheme described in Figure 1 was characterized with marker enzymes, the subcellular distribution of glucuronyl transferase and benzopyrene hydroxylase in trout liver was explored. The results of several distribution experiments focusing on microsomal enzymes are presented in Table 3. Glucose-6-phosphatase, glucuronyl transferase, and benzopyrene hydroxylase had the highest R.S.A. and percent recovery in the microsomal fraction. Similar distribution profiles and R.S.A. were obtained with rotenone-insensitive cytochrome C reductase, another microsomal marker enzyme. However, with NADPH as co-factor, the high R.S.A. of the rotenone-insensitive cytochrome C reductase in the lysosomal fraction indicated significant contamination of this fraction by these enzymes. This was not surprising in light of the report by Sottocasa et al. (1967) that this enzyme also is located in the outer mitochondrial membrane.

In Figure 3 the results of one such distribution experiment are illustrated. The relative specific activity profiles of glucuronyl transferase and benzopyrene hydroxylase are almost superimposable with the profile of glucose-6-phosphatase, a microsomal marker in mammalian liver. Recovery of glucuronyl transferase and benzopyrene hydroxylase in the microsomal fraction was approximately 70%, which was similar to that found for glucose-6-phosphatase.

Although the results indicate that a sharp separation of all organelles was not achieved in all cases, the procedure does allow for adequate resolution of the major subcellular organelles. The non-homogeneity of the lysosomal enzymes seen in this study has been observed in other systems, and, since the major thrust here concerned microsomes, attempts were not made to resolve lysosomes.

The R.S.A. and percent recovery of benzopyrene hydroxylase and glucuronyl transferase were almost identical with these criteria for the microsomal marker, glucose-6-phosphatase. The R.S.A. for rotenone insensitive NADH and NADPH cytochrome C reductase indicates enrichment of this activity in the lysosomal as well as in the microsomal pellet, but these two fractions together contain 70-80% of all microsome markers. Separation of microsomes from plasma membrane has been difficult to achieve in other species, and the relatively high percentage (30-35%) of the plasma membrane markers, alkaline phosphatase and 5'-nucleotidase, in the microsomal fraction indicates that this same prpoblem also exists with trout liver. However, the high R.S.A. of the plasma membrane markers in the nuclear pellet indicates the greatest enrichment on a biochemical basis in this latter fraction.

In several studies concerning the metabolism by liver subcellular fractions of diazinon (Hogan and Knowles 1972), aniline (Pohl et al. 1974), aldrin (Stanton and Khan 1973), and di-2-ethylhexylphthalate (Carter et al. 1974), a considerable amount of activity against these substrates has been found to reside in the "mitochondrial" or 10,000 *g* pellet. The data presented in this study indicate that, even under carefully controlled

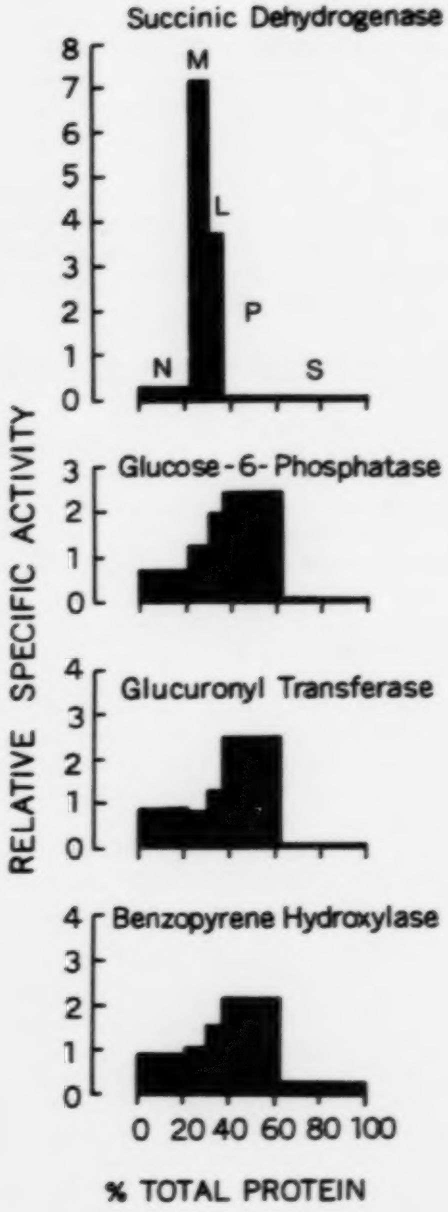

Figure 3. Distribution of mitochondrial and microsomal marker enzymes from trout liver.

fractionation procedures, some microsomes sediment with the mitochondrial fraction and that biotransformation of these compounds by the mitochondrial fraction is most likely associated with contaminating microsomes rather than with mitochondria *per se*. This association is also shown in studies of di-2-ethylhexylphthalate which utilized homogenization in 0.154 *M* KCl rather than the 0.25 *M* sucrose used in this study.

Since microsomes were the only liver subcellular fraction required in the subsequent parts of Section 4 dealing with "hepatic xenobiotic metabolizing activity in rainbow trout", the simplified fractionation procedure described in Section 3 (Methodology Preparation of Subcellular Fractions of Liver) was utilized henceforth.

Effect of Polycyclic Aromatic Hydrocarbons on Hepatic Microsomal Enzymes in Rainbow Trout

Now that the efficacy of the fractionation scheme for obtaining microsomes from trout liver homogenates has been demonstrated, such microsomes can be used in studies to characterize the xenobiotic metabolizing activity of rainbow trout liver. Assays for a variety of microsomal enzyme activities will be validated for use with trout liver microsomes. The presence of these liver microsomal enzyme activities can be evaluated in normal (control) trout and in trout exposed to agents known to affect such enzyme activities in mammals.

The radioactive assays developed for arylhydrocarbon (benzo[a]pyrene) hydroxylase (AHH) and UDPGA-gluruconyl transferase were linear with time and protein concentration under the prescribed condition. The AHH activity in hepatic microsomes of rainbow trout showed characteristics typical of cytochrome P-450 mediated monooxygenation, i.e., the activity was dependent on a source of NADPH and was inhibited by PBO, a classical inhibitor of microsomal monooxygenations. The UDPGA-glucuronyl transferase activity was negligible in the absence of UDPGA but was linear for up to 20 min in the presence of UDPGA.

Three polycyclic aromatic hydrocarbons (β-naphthoflavone, 3-methylcholanthrene, and 2,3-benzanthracene) were investigated for their abilities to induce various microsomal enzyme activities in rainbow trout (Statham et al. 1978). Figure 4 demonstrates that all three polycyclic aromatic hydrocarbons caused dramatic increases in AHH activity without affecting glucose-6-phosphatase or UDPGA-glucuronyl transferase.

Time-course studies for the induction of AHH by the polycyclic aromatic compounds showed a typical time-dependent increase in monooxygenase activity (Figure 5). The AHH activity reached a maximum at about 48 h after injection of 3-methylcholanthrene and 2,3-benzanthracene, but with β-naphthoflavone as the inducer the AHH activity was still increasing 96 h after injection. These differences may be explained by differences in rates of absorption of the various compounds from the intraperitoneal corn oil depot into the vascular system of the fish. Similar observations by Boobis et al. (1977) have been explained in the same manner. However, in mice, it was found that AHH activity returned to basal levels faster after β-

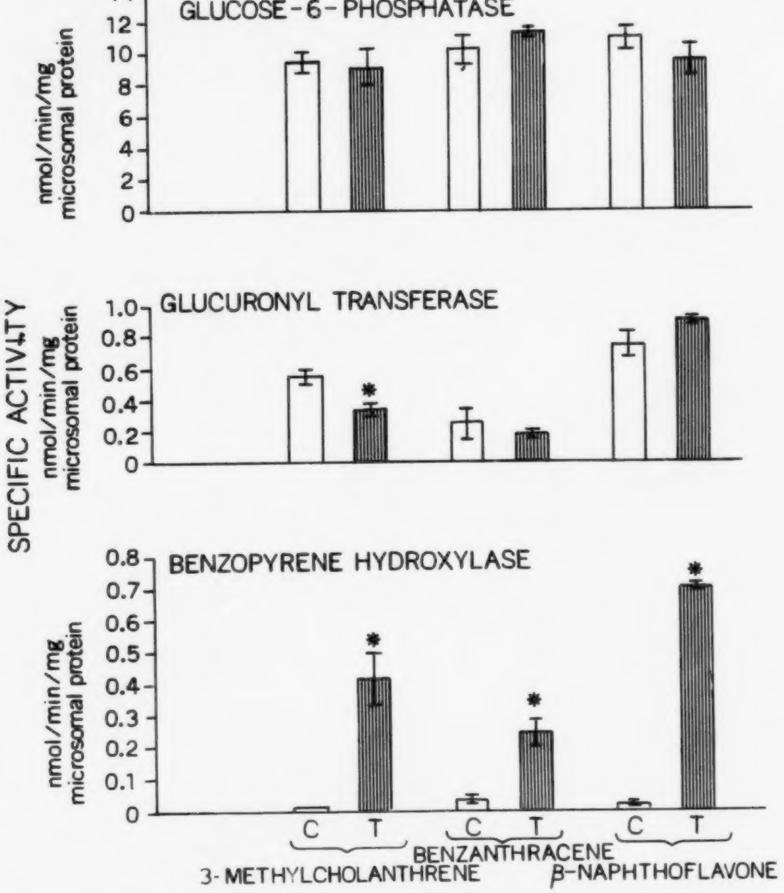

Figure 4. The effect of various inducing agents on selected microsomal enzyme activities. Animals were pretreated as described in Section 3 (Methods). Hepatic microsomes were prepared from individual fish 48 h after dosing. Each bar is the mean ± S.E. (n = 3-5).

* Indicates induced activity (T) significantly different from control activity (C) ($p < 0.05$).

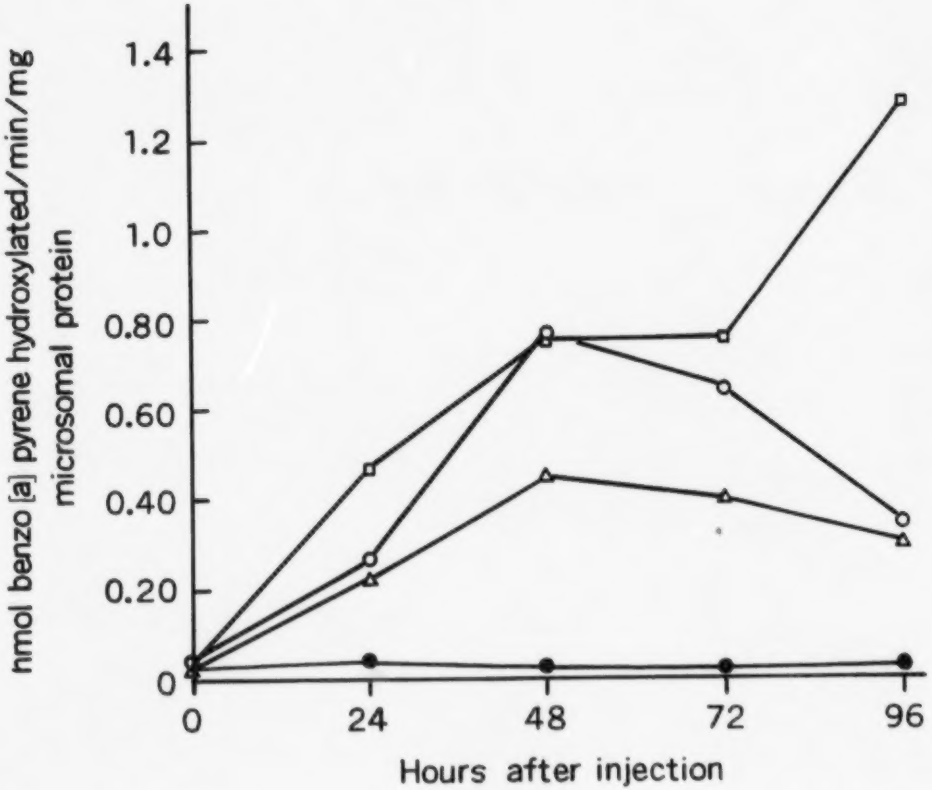

Figure 5. Time-course for induction of arylhydrocarbon (benzo[a]pyrene) hydroxylase by various polycyclic hydrocarbons. Each point is the mean of two separate experiments each using the pooled livers from two fish. ●—● , corn oil control; △—△, 2,3-benzanthracene; ○—○ , 3-methylcholanthrene; □—□, β-naphthoflavone.

naphthoflavone pretreatment than after injections of 3-methylcholanthrene (Boobis et al. 1977).

The induction of AHH by 3-methylcholanthrene, β-naphthoflavone, and 2,3-benzanthracene was accompanied by an increase in levels of hepatic microsomal cytochrome P-450 (Table 4). The P-450 hemoprotein concentrations of the induced microsomes were approximately 50% higher than those of the control microsomes.

TABLE 4. CYTOCHROME P-450 CONTENT OF VARIOUSLY-INDUCED TROUT HEPATIC MICROSOMES

Pretreatment of fish	Dose, mg/kg	Cytochrome P-450, nmol/mg of protein
Corn oil (control	1	0.210; 0.223[a]
β-Naphthoflavone	100	0.334; 0.354[a]
3-Methylcholanthrene	20	0.311; 0.337[a]
2,3-Benzanthracene	10	0.336; 0.277[a]

[a] Values obtained from pooled microsomes (6-8 fish/group) in two separate experiments. Microsomes prepared 48 h after treatment of fish.

Polycyclic aromatic hydrocarbon inducing agents have been shown previously to induce AHH activity in several marine species (Bend et al. 1973, James et al. 1977); in these studies, however, no concomitant increase in cytochrome P-450 levels were observed. In the present studies--using rainbow trout--increases in cytochrome P-450 concentration in response to β-naphthoflavone, 3-methylcholanthrene, and 2,3-benzanthracene were found. This increase in cytochrome P-450 levels may reflect differences in the control mechanisms of P-450 systhesis of marine and freshwater species of fish.

No distinctive alterations in the position of the Soret peak of the CO complex of the $NaSO_2O_4$-reduced hemoprotein was noted after induction by the polycyclic hydrocarbons. This finding contrasts with the situation in rats and other mammals where a shift from 450 to 448 nm was observed (Sladek and Mannering 1966).

Further evidence for a true induction, rather than an activation of existing enzymes, was found in *in vitro* studies. The inclusion of β-naphthoflavone in the AHH assay, or the preincubation of control microsomes with β-naphthoflavone and NADPH prior to the AHH assay, did not increase the hydroxylation of benzo[a]pyrene. The data in Table 5 demonstrate that β-naphthoflavone was actually inhibitory to the AHH assay.

The observation that β-naphthoflavone, added *in vitro*, inhibited the AHH activity of control microsomes suggests that the endogenous cytochrome P-450 of trout hepatic microsomes may be of the P_1-450 type. This suggestion seems likely because in mammalian systems β-naphthoflavone actually stimulates P-450 mediated AHH while inhibiting P_1-450 mediated AHH activity (Wiebel et al. 1971). Furthermore, no blue shift in the absorbance maximum of the CO complex of the ferrocytochrome was seen after induction, which suggest that the induced and endogenous cytochrome(s) are similar.

TABLE 5. *IN VITRO* EFFECT OF β-NAPHTHOFLAVONE
ON ARYLHYDROCARBON (BENZO[A]PYRENE) HYDROXYLASE
ACTIVITY OF HEPATIC MICROSOMES IN CONTROL TROUT

Addition to assay		AHH activity[a], %
None		100
Dimethylformamide	5 μliter	98.3
β-Naphthoflavone[b]	10 μ*M*	65.3
	100 μ*M*	46.3
	500 μ*M*	22.6

[a] nmol of polar metabolites of benzo[a]pyrene produced/min/mg microsomal protein.
[b] β-Naphthoflavone was added dissolved in 5 μl of *N,N*-dimethylformamide.

These differences between the induction of hepatic microsomal cytochrome(s) P-450 in fish and in mammals indicated the need for a more detailed study of induction and cytochrome(s) P-450 in rainbow trout.

Induction and Characterization of Cytochrome(s) P-450 and Monooxygenation in Rainbow Trout

The four model monooxygenase assays were validated in rainbow trout hepatic microsomes (Elcombe and Lech 1979). Temperature optima of 25-26°C were found for ethylmorphine-*N*-demethylation and benzo[a]pyrene hydroxylation, while for ethoxyresorufin- and ethoxycoumarin-*O*-deethylations the optima were 29-31°C. All reaction velocities were calculated over the linear portion of the reaction. The dependence of the reaction velocities on the concentration of microsomal protein was also examined. These results are depicted in Figure 6. These parameters were constant for all rainbow trout hepatic microsomal preparations examined.

Pretreatment of rainbow trout with the polyhalogenated biphenyls (Firemaster BP6, Aroclor 1242, or Aroclor 1248) had no effect on the liver/body ratios or on the yields of microsomal protein per unit wet weight of liver (Table 6) (Elcombe and Lech 1973). However, PBBs and PCBs were able to induce the activity of certain monooxygenase reactions.

Dose-response studies of the induction process indicated maximal induction of PBBs and PCBs at about 250 mg/kg (Figure 7 and Figure 8).

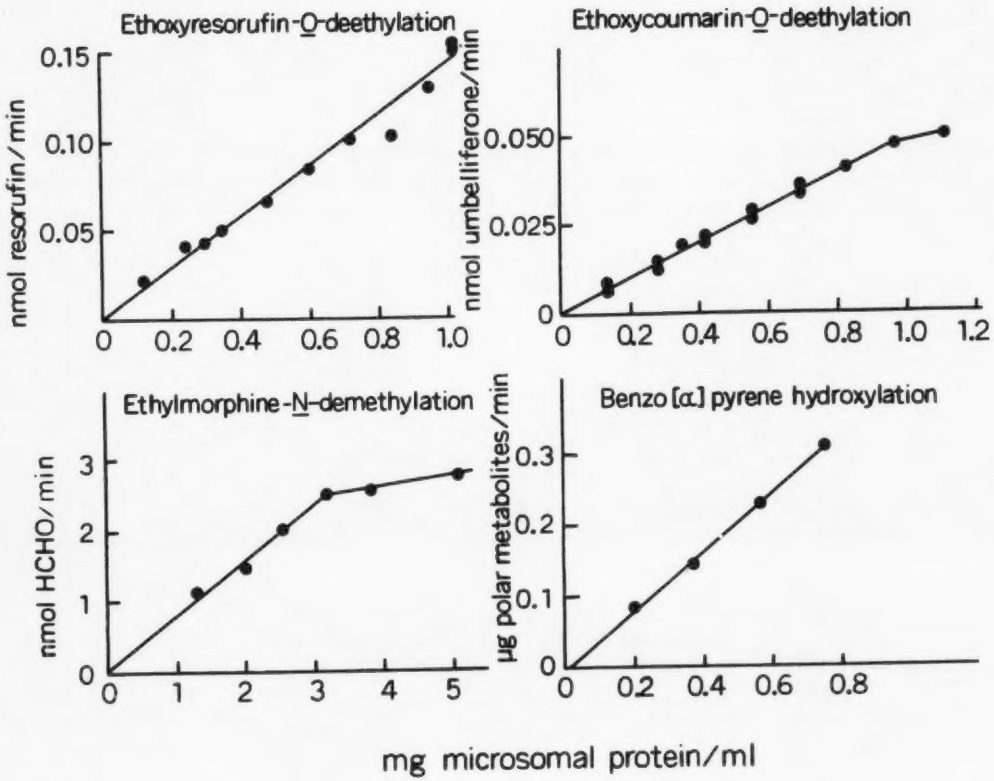

Figure 6. Protein dependency for various monooxygenations in rainbow trout hepatic microsomes.

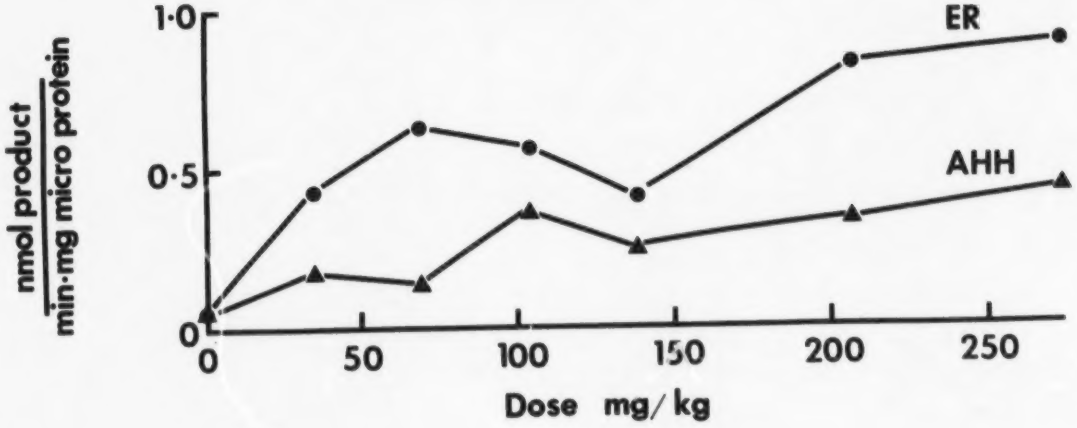

Figure 7. Dose-response relationship for Aroclor 1242. Each point represents values from microsomes observed from the pooled livers of six fish. Values were determined 4 days after injection. ●–● ethoxyresorufin-*O*-deethylation; ▲–▲ arylhydrocarbon hydroxylase.

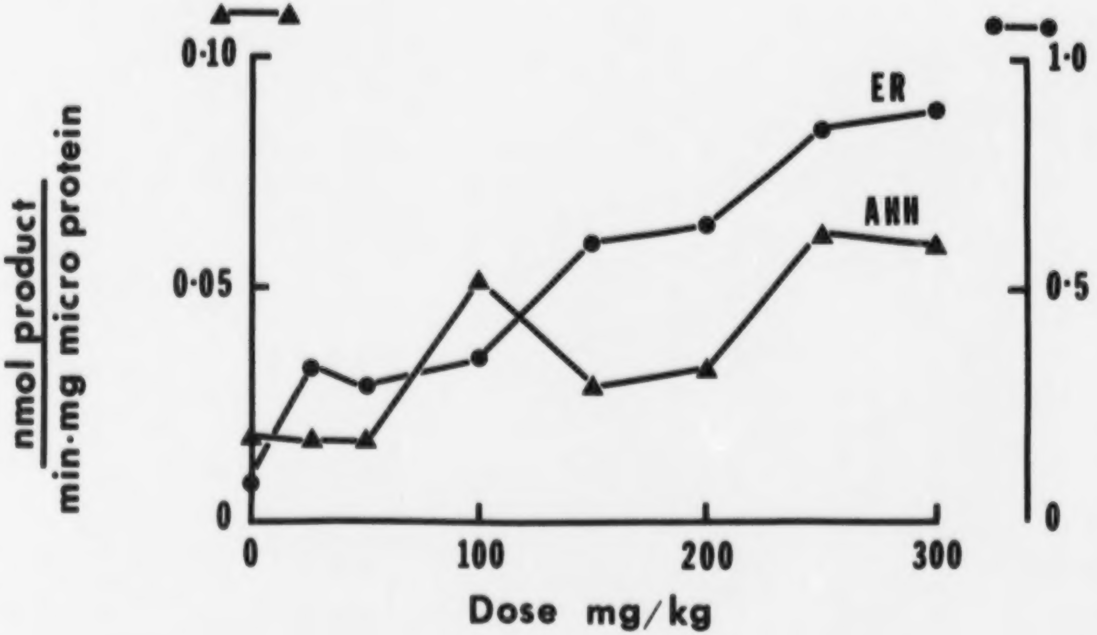

Figure 8. Dose-response relationship for Firemaster BP6. Each point represents values from microsomes obtained from the pooled liver of six fish. Values were determined 4 days after injection. ●—● ethoxyresorufin-*O*-deethylation; ▲—▲ arylhydrocarbon hydroxylase.

TABLE 6. LIVER:BODY WEIGHT RATIOS AND YIELD OF MICROSOMAL PROTEIN[a]

Treatment	Dose	Liver:body ratio, %	mg microsomal protein/g liver
Corn oil	1 ml/kg	0.96 ± 0.05	24.5 ± 0.8
Firemaster BP6	150 mg/kg	1.07 ± 0.04	24.0 ± 0.5
Aroclor 1254	150 mg/kg	0.92 ± 0.03	25.0 ± 1.6
Aroclor 1242	150 mg/kg	0.96 ± 0.06	23.6 ± 2.7

[a] Values are mean ± SE (n=6).

Aroclor 1242 maximally induced arylhydrocarbon (benzo[a]pyrene) hydroxylase (AHH) activity by approximately 10-fold, while PBBs resulted in only a 4-fold increase. Ethoxyresorufin-*O*-deethylase was maximally stimulated by approximately 30- and 20-fold by PBBs and Aroclor 1242 respectively.

Studies of the time-course of induction for Aroclors 1242 and 1254 at a dose of 150 mg/kg indicated that maximal stimulation of monooxygenation was attained after 4 and 7 days respectively (Figure 9 and Figure 10). A single intraperitoneal injection of Aroclor 1242 elevated AHH, ethoxycoumarin-*O*-deethylase, and ethoxyresorufin-*O*-deethylase by about 10-fold at 4 days post-injection. These enzyme activities remained elevated for at least 15 days after treatment of the fish (Figure 9). Figure 10 shows a similar 10-fold induction of monooxygenase activity by Aroclor 1254. Stimulation of monooxygenation was still apparent 21 days after treatment.

Ethylmorphine-*N*-demethylation was unaffected by pretreatment of fish with Aroclors 1254 or 1242. This finding is illustrated for Aroclor 1254 in Figure 10.

After injection of rainbow trout with Firemaster BP6, no increases in monooxygenase activities were seen until 3 days after treatment (Figure 11). After this time, ethoxyresorufin- and ethoxycoumarin-*N*-deethylations remained elevated for at least 2 weeks, although AHH declined rapidly to reach control levels about 7 days after treatment of the fish. Ethoxycoumarin-*O*-deethylation did not reach a maximum until 7 days after injection. This delay has also been observed in rats treated with Firemaster BP6 (Dent, et al. 1978).

In common with the PCBs, PBBs also failed to increase ethylmorphine-*N*-demethylation above control values. In general Aroclor 1254 and Aroclor 1242 appeared to be more potent (in terms of mg/kg) than Firemaster BP6; however, the latter compound was much more effective at inducing ethoxyresorufin-*O*-deethylation.

The stimulatory effect of PCBs and PBBs on monooxygenations appears to be a true induction since inclusion of these compounds in the *in vitro* assays had no effect upon the observed enzymatic activities.

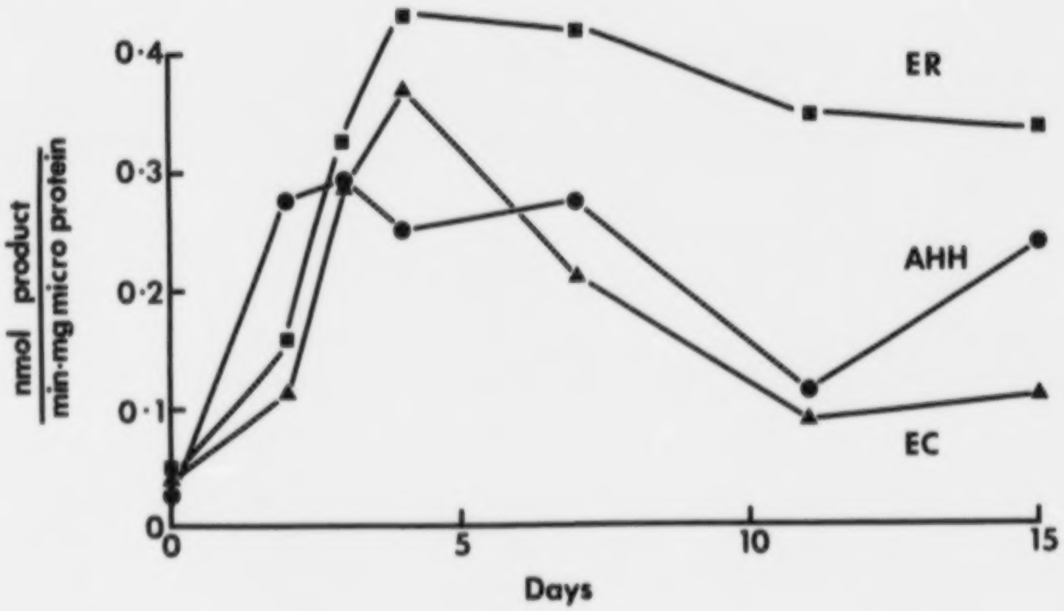

Figure 9. Time course of induction by Aroclor 1242. Each point represents values from microsomes obtained from the pooled liver of six fish. Aroclor 1242 was administered intraperitoneally in corn oil at a dose of 150 mg/kg. ■—■ ethoxyresorufin-*O*-deethylation; ▲—▲ ethoxycoumarin-*O*-deethylation; ●—● arylhydrocarbon hydroxylation.

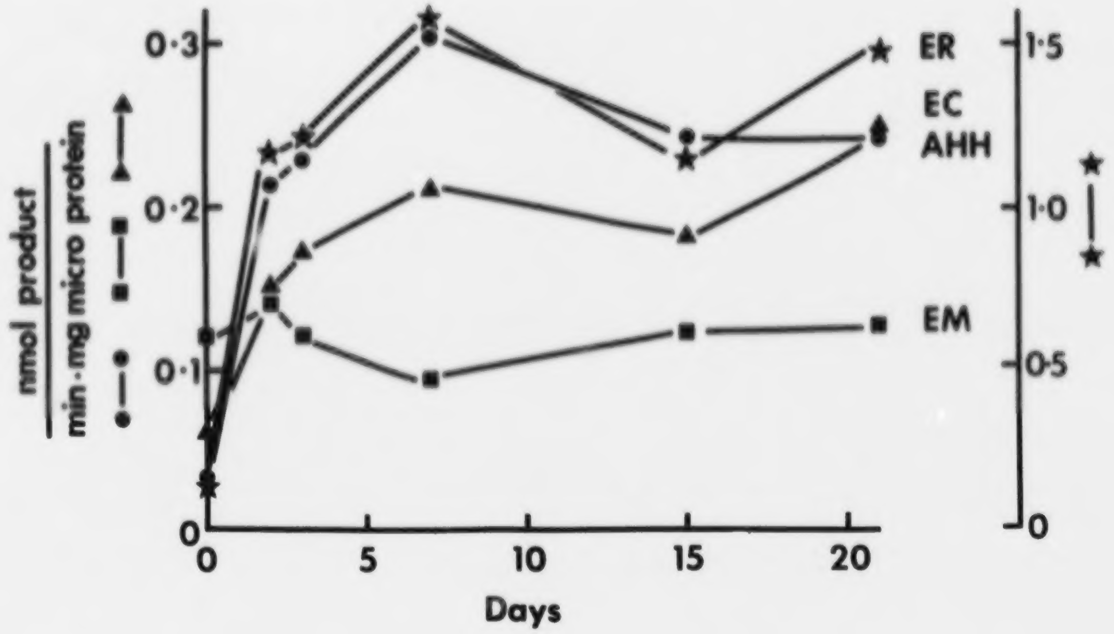

Figure 10. Time-course for induction by Aroclor 1254. Each point represents values from microsomes obtained from the livers of six fish. Aroclor 1254 was administered intraperitoneally in corn oil at a dose of 150 mg/kg. ★—★ ethoxyresorufin-*O*-deethylation; ▲—▲ ethoxycoumarin-*O*-deethylation; ●—● arylhydrocarbon hydroxylase; ■—■ ethylmorphine-*N*-demethylase.

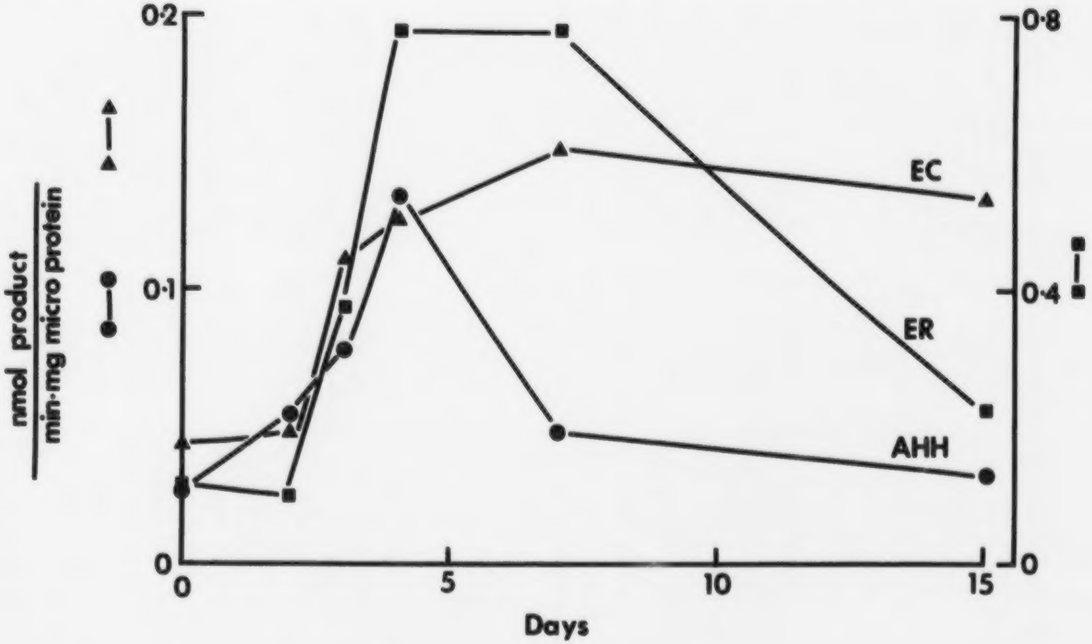

Figure 11. Time-course for induction by Firemaster BP6. Each point represents values from microsomes obtained from the pooled liver of six fish. Firemaster BP6 was administered intraperitoneally at a dose of 150 mg/kg. ■—■ ethoxyresorufin-*O*-deethylation; ▲—▲ ethoxycoumarin-*O*-deethylation; ●—● arylhydrocarbon hydroxylase.

Hemoprotein P-450 concentrations in rainbow trout hepatic microsomes were determined 4 days after pretreatment with various inducing agents. Small increases (10-20%) in hemoprotein P-450 content were observed after treatment of fish with PCBs or PBBs, but these increases were not statistically significant (Table 7). However, pretreatment of fish with β-naphthoflavone resulted in a significant 40% increase in the level of P-450 hemoprotein.

TABLE 7. CYTOCHROME P-450 CONTENT AND 455/430 PEAK RATIOS OF EtNC SPECTRA

Treatment	Dose	P-450 nmol/mg protein	EtNC spectra 455/430 ratio
Corn oil	1 ml/kg	0.118 ± 0.003	0.28 ± 0.01
Phenobarbital	65 mg/kg	0.111 ± 0.006	0.32 ± 0.04
Firemaster BP6	150 mg/kg	0.132 ± 0.014	0.37 ± 0.05
Aroclor 1254	150 mg/kg	0.143 ± 0.020	0.35 ± 0.05
Aroclor 1242	150 mg/kg	0.137 ± 0.001	0.39 ± 0.01
β-naphthoflavone	100 mg/kg	0.165 ± 0.005[a]	0.40 ± 0.10

[a] Significantly different from corn oil control group, $p < 0.05$, $n = 3$. Each determination used pooled microsomes from the livers of two fish. Values are mean ± SE.

Significantly, the λ_{max} for the CO complex of reduced cytochrome P-450 was at 449 nm in all preparations examined (Figure 12).

The interaction of ethylisocyanide with $Na_2S_2O_4$ reduce cytochrome P-450 resulted in optical difference spectra with absorption maxima at 432-433 nm and 453-454 nm in all microsomal preparations examined. A small increase in the 455:430 peak ratio was observed after pretreatment with PBBS, PCBs, or β-naphthoflavone (Table 7).

The inducing properties of the polyhalogenated biphenyls appear to be expressed differently in fish than in rodents. The polyhalogenated biphenyls failed to increase liver:body weight ratios or yield of microsomal protein; furthermore, increases in ethylmorphine-*N*-demethylase were not apparent. These responses are typical of polyhalogenated biphenyl induction in rodents (Ecobiochon and Comeau 1974, Bickers et al. 1975, Goldstein et al. 1975, Dent et al. 1976a, 1976b, 1977a) and are characteristic of cytochrome P-450 induction caused by phenobarbitals. PCB and PBB treatment of rainbow trout were found to stimulate AHH, ethoxycoumarin-*O*-deethylation, and ethoxyresorufin-*O*-deethylation. AHH and ethoxycoumarin-*O*-deethylation are induced by both phenobarbital and 3-methylcholanthrene in rodents; however, ethoxyresorufin-*O*-deethylation is specifically inducible by the polyclic aromatic hydrocarbon type of inducing agents (cytochrome P_1-450) (Burke and Mayer 1974).

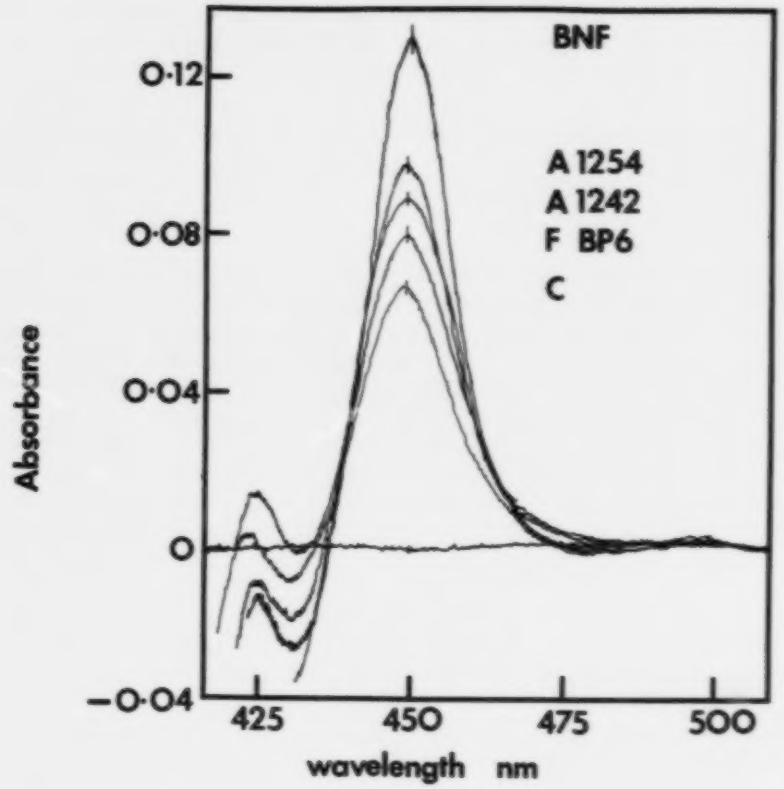

Figure 12. Hemoprotein P-450 difference spectra. Microsomes (24 mg/ml) were divided between two cuvettes. A baseline of equal light absorbance was obtained and CO was bubbled through each cuvette. A few mg of $Na_2S_2O_4$ were added to the sample cuvette, and the resultant spectrum between 420 and 510 nm was recorded. β-naphthoflavone (BNF) (100 mg/kg); Aroclor 1254 (A1254) (150 mg/kg); Aroclor 1242 (A1242) (150 mg/kg); Firemaster BP6 (FBP6) (150 mg/kg); corn oil (C) (1 ml/kg).

Because of the ability of commercial polyhalogenated biphenyls to elicit both phenobarbital-like and 3-methylcholanthrene, "mixed" inducer has frequently been used. Other studies have demonstrated that cytochrome P_1-450 or cytochrome P-450 induction by PCBs is determined by the substitution pattern of the halogen atoms (Goldstein et al. 1977, Poland and Glover 1977). More recently, Moore and Aust (1977) and Moore et al. (1977) have suggested that a similar situation exists with the PBBs.

In contrast to the apparent "mixed" patterns of stimulation of monooxygenation in rodents due to polyhalogenated biphenyls, rainbow trout seem incapable of responding to induction of cytochrome P-450 related to phenobarbitals.

The large increases (up to 25-30 fold) in monooxygenase activity observed after PCB or PBB treatment of fish cannot be explained in terms of an increase in concentrations of total hemoprotein P-450, since the levels of this (these) enzyme(s) were increased by only about 10-20%. Hence, a novel enzyme with different substrate specificity is possibly induced by the PBBs and PCBs in rainbow trout. However, no changes in the wavelength of the absorption maximum of the CO complex of ferrocytochrome P-450 were seen, nor were the ratios of the 455:430 nm absorption maxima of the ethylisocyanide complex of ferrocytochrome P-450 significantly altered. In rodents the 455:430 peak ratio increases considerably after treatment with PCBs or PBBs (Alvares et al. 1973, Goldstein et al. 1977, Dent et al. 1977).

Dose-response studies for induction of arylhydrocarbon (benzo[a]pyrene) hydroxylation by β-naphthoflavone indicated that maximal induction was obtained using a dose of 100 mg/kg (Figure 13) (Elcombe and Lech 1979).

To obtain more detailed biochemical results on the properties of induced hemoprotein P-450-mediated reactions, β-naphthoflavone and Aroclor 1242 were used in doses of 100 and 150 mg/kg, respectively. The dose of phenobarbital was 65 mg/kg since preliminary studies indicated that a dose of 80 mg/kg was toxic to a majority of fish. Animals were sacrificed and microsomes obtained 3 days after injection.

Table 8 demonstrates that none of the pretreatments had effects upon either the liver:body weight ratio or the yield of microsomal protein obtained from rainbow trout liver.

Hepatic microsomal ethylmorphine-*N*-demethylation was unaffected by pretreatment of rainbow trout with β-naphthoflavone, Aroclor 1242, or phenobarbital; however, arylhydrocarbon (benzo[a]pyrene) hydroxylation was increased 10- and 40-fold by Aroclor 1242 and β-naphthoflavone, respectively. Phenobarbital had no apparent effect upon arylhydrocarbon hydroxylase activity (Table 9).

Michaelis-Menten kinetics for the deethylations of ethoxycoumarin and ethoxyresorufin were examined in the variously-induced trout hepatic microsomal preparations. Figure 14 indicates the biphasic nature of the Lineweaver-Burk plots obtained for ethoxycoumarin-*O*-deethylation in microsomes from β-naphthoflavone, Aroclor 1242 pretreated, and control

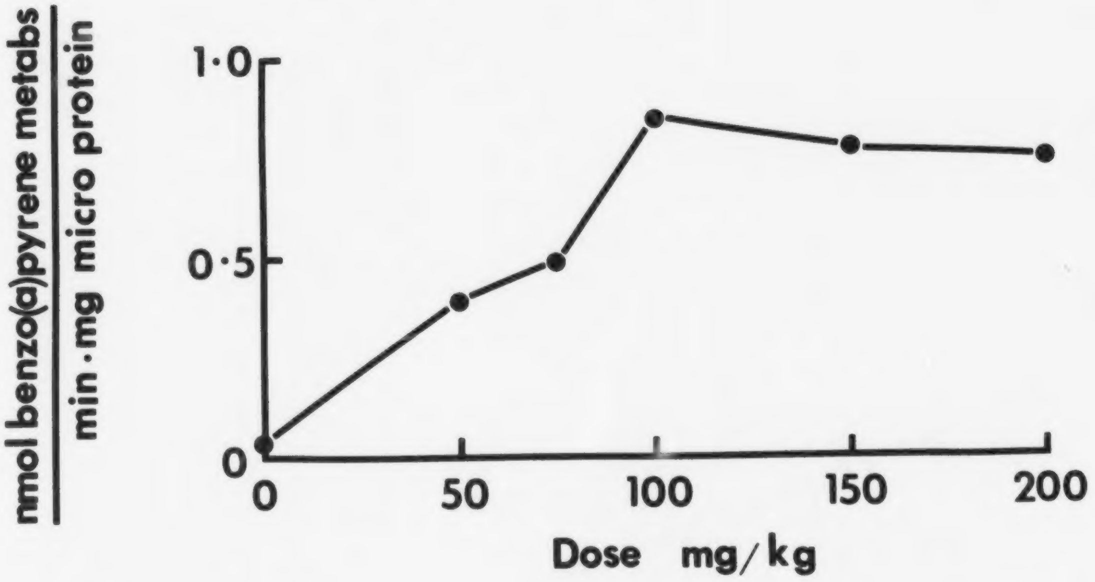

Figure 13. Dose-response relationship for β-naphthoflavone induction of benzo[a]pyrene hydroxylation in rainbow trout.

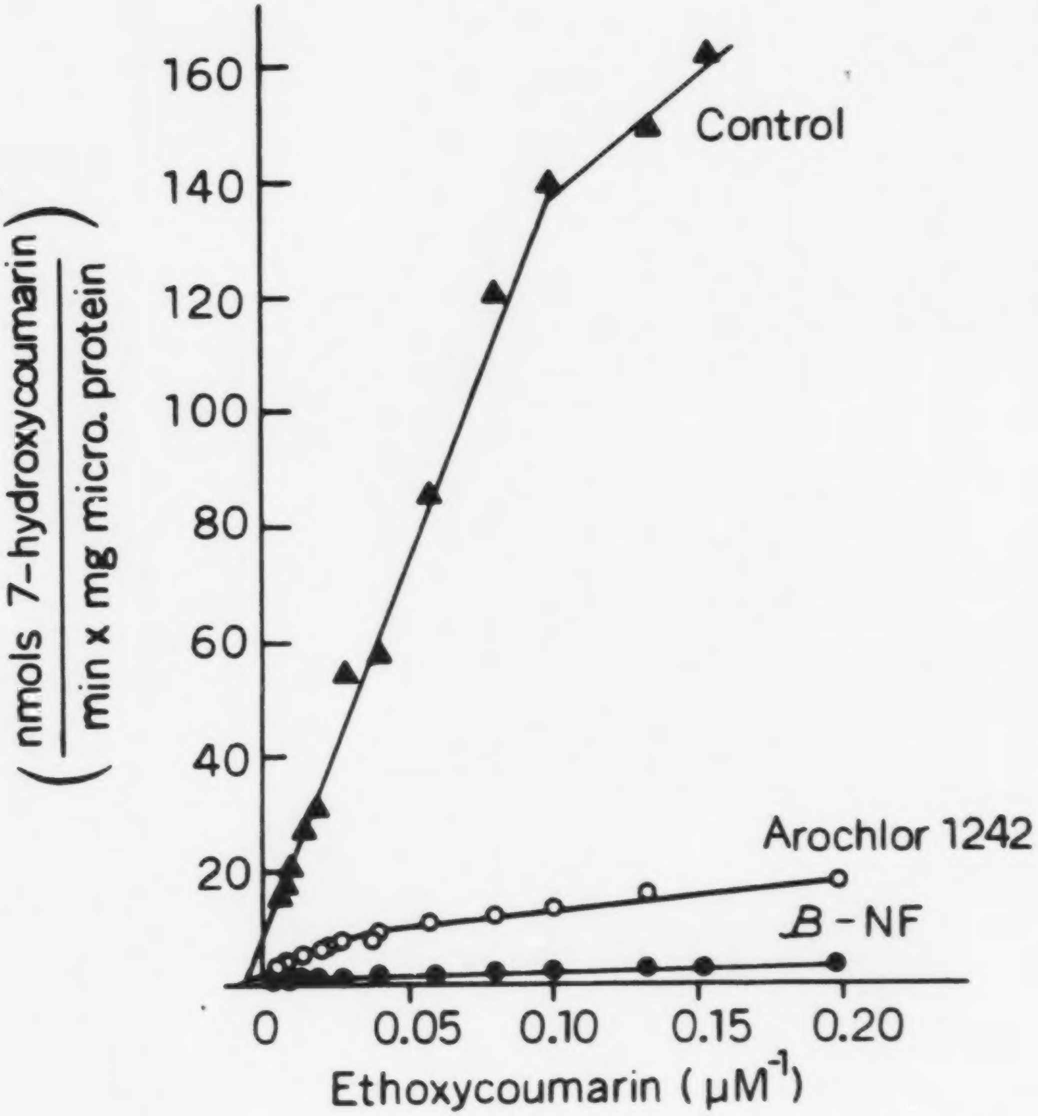

Figure 14. Lineweaver-Burk plots for ethoxycoumarin-*O*-deethylation by rainbow trout hepatic microsomes. β-NF is β-naphthoflavone. ▲-▲, Corn oil-treated fish; ○-○, Aroclor 1242-treated fish; ●-●, β-naphthoflavone-treated fish.

TABLE 8. MICROSOMAL YIELDS AND LIVER:BODY RATIOS AFTER PRETREATMENT OF RAINBOW TROUT WITH VARIOUS INDUCING AGENTS[a]

Pretreatment	Dose	Microsomal yield, mg protein/g liver	Liver/body ratio, %
Corn oil	1 ml/kg	14.21 ± 0.38	0.99 ± 0.03
Aroclor 1242	150 mg/kg	15.00 ± 2.33	0.99 ± 0.05
β-Naphthoflavone	100 mg/kg	13.49 ± 0.37	1.00 ± 0.01
Phenobarbital	65 mg/kg	14.95 ± 0.04	0.96 ± 0.08

[a] Values were determined 72 h after injection of fish and are mean ± SE.

TABLE 9. INDUCTION OF MONOOXYGENATION IN RAINBOW TROUT HEPATIC MICROSOMES FOLLOWING INTRAPERITONEAL PRETREATMENT

Pretreatment	Ethylmorphine-*N*-demethylase		Benzo[a]pyrene hydroxylase	
	nmol/min/mg	% control	nmol/min/mg	% control
Corn oil 1 ml/kg	0.768 ± 0.054	100	0.022 ± 0.003	100
Aroclor 1242 150 mg/kg	0.753 ± 0.067	98	0.233 ± 0.012[a]	1059
β-Naphthoflavone 100 mg/kg	0.678 ± 0.137	88	0.898 ± 0.119[a]	4081
Phenobarbital 65 mg/kg	0.622 ± 0.095	81	0.023 ± 0.004	104

[a] Significantly different from control (corn oil) group. Values were determined 72 h after injection of fish and are mean ± SE.

trout. In Figure 15, the ordinate has been expanded for clarity. The double reciprocal plot obtained for the deethylation of ethoxycoumarin by phenobarbital-microsomes was identical to that of the control microsomes.

Ethoxyresorufin-*O*-deethylation depicted typical linear Lineweaver-Burk plots in all cases (Figure 16).

Table 10 summarizes the kinetic parameters obtained for the *O*-deethylation reactions in variously-induced microsomes. The apparent Km values for ethoxyresorufin-*O*-deethylations (about 150 μ*M*) were unchanged by pretreatment of the rainbow trout. However, pretreatment with Aroclor 1242 and β-napthoflavone resulted in 13- and 44-fold increases in the apparent V_{max} values for the reaction. β-Napthoflavone and Aroclor 1242 increased the apparent V_{max} values for ethoxycoumarin-*O*-deethylation by about 11- and 3-fold, respectively; these agents also decreased the apparent Km values from 129 to about 50 μ*M*.

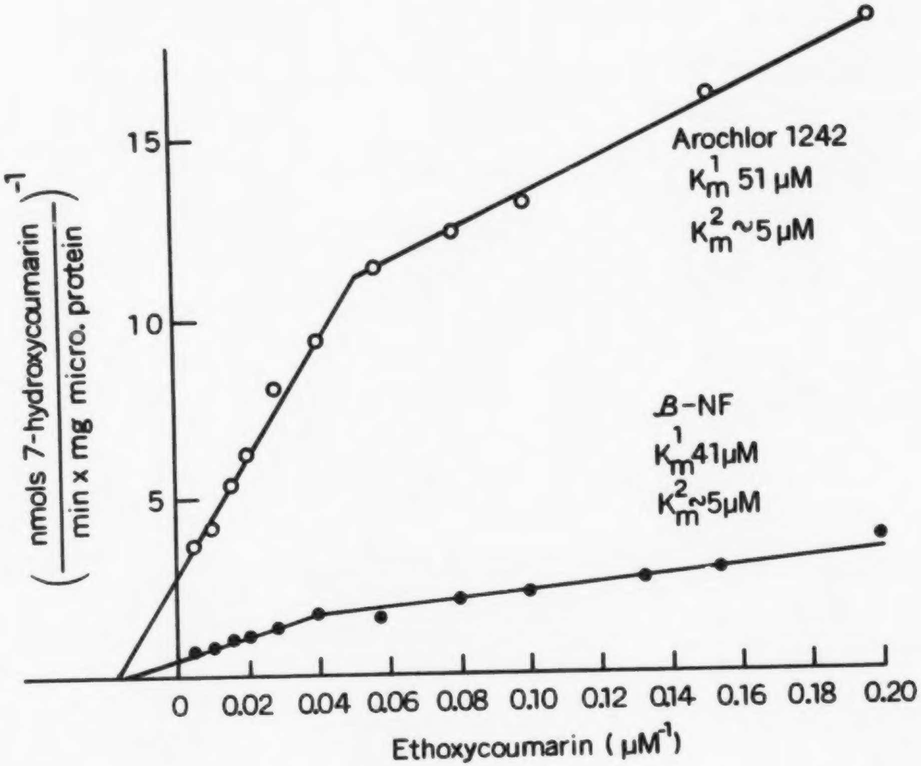

Figure 15. Lineweaver-Burk plots for ethoxycoumarin-*O*-deethylation by rainbow trout hepatic microsomes with expanded ordinate to demonstrate biphasic nature. β-NF is β-naphthoflavone.

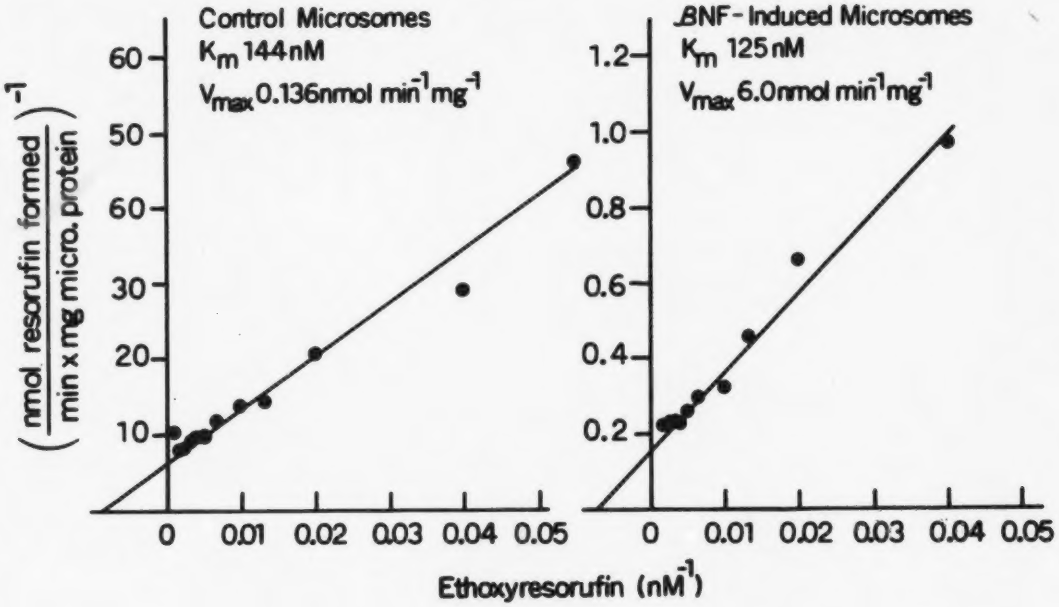

Figure 16. Lineweaver-Burk plots for ethoxyresorufin-*O*-deethylation by rainbow trout hepatic microsomes. β-NF is β-naphthoflavone.

TABLE 10. THE EFFECT OF INDUCERS ON THE KINETICS OF MONOOXYGENATION IN RAINBOW TROUT HEPATIC MICROSOMES FOLLOWING INTRAPERITONEAL PRETREATMENT

	Ethoxyresorufin-*O*-deethylase			Ethoxycoumarin-*O*-deethylase		
Pretreatment	Vmax nmol/min/mg	% control	Km, n*M*	Vmax nmol/min/mg	% control	K_m, n*M*
Corn oil 1 ml/kg	0.136±0.026[a]	100	144±6	0.101±0.010	100	129±9
Aroclor 1242 150 mg/kg	1.85±0.04[b]	1367	154±0	0.286±0.047[a]	283	51±1[b]
β-Naphthoflavone 100 mg/kg	6.06±0.18[b]	4455	125±8	1.19±0.28[a]	1178	41±4[b]
Phenobarbital 65 mg/kg	0.088±0.017	65	170±0	0.065±C.017	64	105±5

[a] Values are mean ± SE; all values obtained 72 h after injection of fish.

[b] Significantly different from corn oil control group, $P < 0.05$.

Inhibitor Studies

Additional information on the nature of the cytochrome(s) P-450 present in trout liver microsomes can be gained through use of inhibitors *in vitro*. Previous studies in rodents, have shown that is is possible by the differential inhibition of microsomal monooxygenation to characterize cytochrome(s) P-450 subpopulations. β-Naphthoflavone, a potent inhibitor of cytochrome P -450 in rodents, almost completely abolished arylhydrocarbon (benzo[a]pyrene) hydroxylation in microsomes obtained from control, β-naphthoflavone-, Aroclor 1242-, and phenobarbital-pretreated trout (Table 11). Metyrapone, an inhibitor of cytochrome P-450 in rodents, had little effect on the arylhydrocarbon hydroxylase activity in control, β-naphthoflavone, Aroclor 1242, or phenobarbital microsomes.

The hemoprotein P-450 content of hepatic microsomes from control fish was 0.234 nmol/mg protein and the absorbance maximum of the carboxyferrocytochrome complex was at 449 nm. β-Naphthoflavone and Aroclor 1242 increased the specific content of P-450 to 0.341 and 0.261 nmol/mg protein, respectively (Table 12); however, these agents did not affect the position of the Soret absorbance maximum (Figure 12). Phenobarbital had no effect on these parameters.

Ethylisocyanide (EtNC) reacts with ferrocytochrome(s) P-450 to produce a complex that exhibits absorbance maxima at about 430 and 455 nm. The ratio of these peaks has been used to distinguish between different forms of hemoprotein(s) P-450. A slight increase in the 455:430 ratios was observed after pretreatment of trout with Aroclor 1242 and β-naphthoflavone

TABLE 11. THE *IN VITRO* EFFECTS OF β-NAPHTHOFLAVONE AND METYRAPONE ON ARYLHYDROCARBON (BENZO[a]PYRENE) HYDROXYLASE IN HEPATIC MICROSOMES OF VARIOUSLY-PRETREATED RAINBOW TROUT

Inhibitor	Concentration, μ*M*	Pretreatment of fish Corn oil	 Phenobarbital	 Aroclor 1242	 β-Naphthoflavone
None	-	100[b] (0.024)[c]	100 (0.026)	100 (0.757)	100 (1.003)
Metyrapone	10	96	92	116	96
	100	100	77	85	88
	500	85	84	75	84
α-Naphthoflavone	10	19	23	22	9
	100	15	14	9	2
	500	12	9	9	1

[a] Inhibitors were added dissolved in 5 μliter *N, N*-dimethylformamide. This solvent had less than a 10% effect on the enzymic activity.

[b] Values are percentage of remaining activity.

[c] Values in parenthesis are activity expressed as nmol/min/mg protein.

TABLE 12. CYTOCHROME P-450 CONTENT AND 455:430 PEAK RATIOS OF EtNC SPECTRA

Pretreatment	Dose	EtNC Spectra, 455:430 ratio	P-450, nmol/mg protein[a]
Corn oil	1 ml/kg	0.28 ± 0.01	0.234 ± 0.006
Phenobarbital	65 mg/kg	0.32 ± 0.04	0.222 ± 0.010
Aroclor 1242	150 mg/kg	0.39 ± 0.01	0.261 ± 0.001
β-Naphthoflavone	100 mg/kg	0.44 ± 0.10	0.341 ± 0.010[b]

[a] Assuming extinction coefficient of 100 mM^{-1} cm^{-1}.

[b] Significantly different from corn oil control group, $p < 0.05$. Values are mean ± SE, n = 3; each determination used pooled microsomes from the livers of two fish.

(Table 12); however, the increase was not statistically significant at the level of significance chosen in this study.

This study, in contrast with previous reports, demonstrated that type I and type II compounds would elicit binding spectra with rainbow trout hepatic microsomal hemoprotein(s) P-450. Figure 17 and Figure 18 illustrate

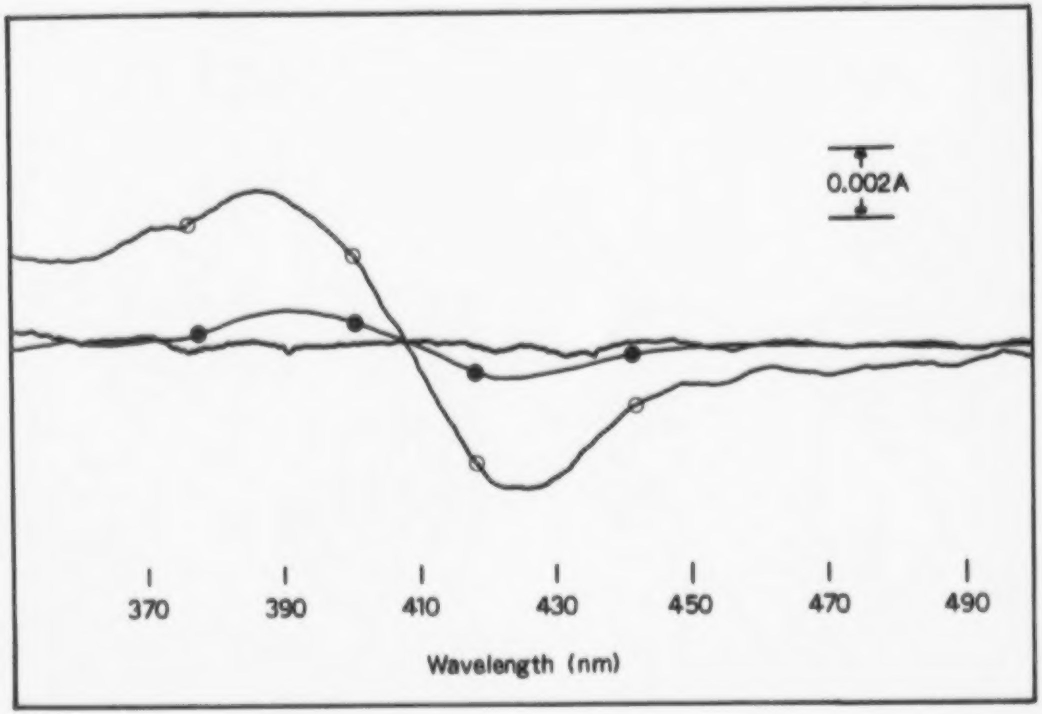

Figure 17. Type I substrate binding spectra of hexobarbital and piperonyl butoxide with rainbow trout hepatic microsomes. Microsomal suspension from control fish (approximately 2 mg/ml) were divided between two cuvettes and a baseline of equal light absorbance obtained between 500 and 350 nm. Piperonyl butoxide (o—o) or hexobarbital (●—●) were added to the sample cuvette at a final concentration of 833 µM. The spectrum was recorded again between 500 and 350 nm.

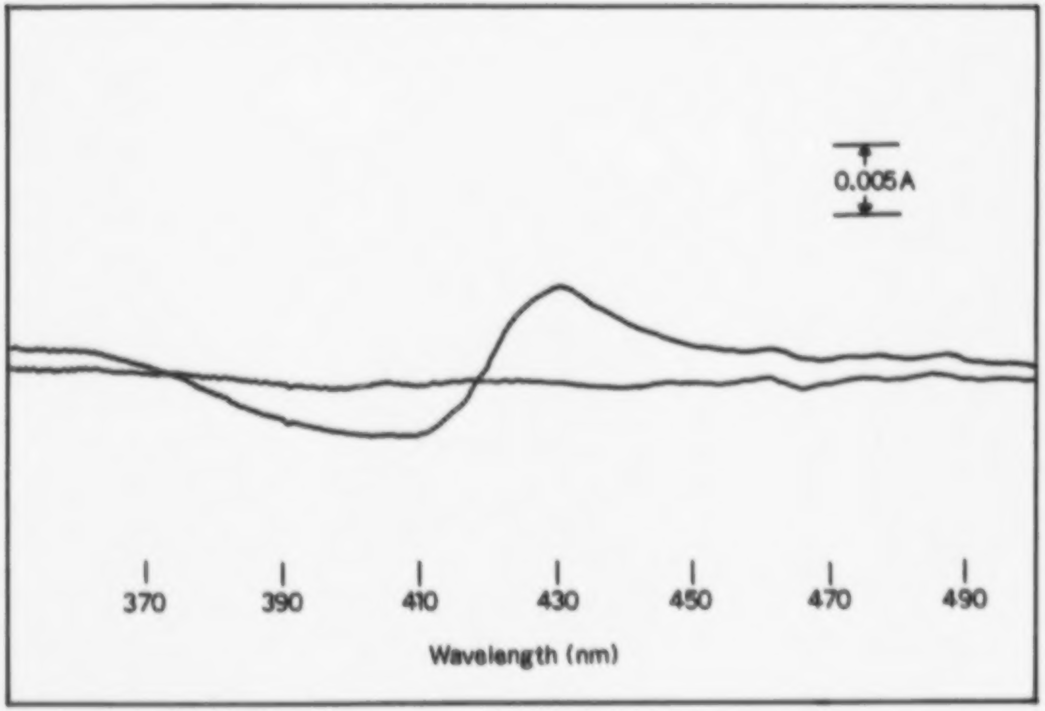

Figure 18. Type II ligand binding spectrum of imidazole with rainbow trout hepatic microsomes. Conditions as in Figure 5, except imidazole was added to a final concentration of 833 µM.

typical binding spectra for PBO (type I), hexobarbital (type I), and imidazole (type II).

Furthermore, the formation of methylenedioxyphenyl metabolic intermediate-ferrohemoprotein P-450 complexes, which is seen with rodent microsomes, also was observed using rainbow trout hepatic microsomes (Figure 19).

Uptake and Elimination of Phenobarbital from Liver

The lack of effect of phenobarbital upon monooxygenase activity could be due to poor adsorption of the compound from the intraperitoneal cavity. However, studies utilizing ^{14}C-phenobarbital suggested that this was not the case (Figure 20). Uptake of the compound was rapid, and elimination of radioactivity followed a time-course similar to that observed in rodents.

Effect of Pretreatment Agents upon *In Vitro* Arylhydrocarbon Hydroxylation—

The observed increases in monooxygenase activity after pretreatment of trout with β-naphthoflavone or Aroclor 1242 possibly was caused by an activation rather than an induction phenomenon. Similarly, the lack of response to phenobarbital may have been due to inhibition of monooxygenation by the barbiturate, as suggested by previous studies (Bend et al. 1973). However, studies of arylhydrocarbon (benzo[a]pyrene) hydroxylation in the presence of various concentrations of these agents (10-500 μ*M*) did not support these suggestions. Table 13 illustrates that Aroclor 1242 had little effect upon monooxygenation *in vitro* but that β-naphthoflavone dramatically inhibited the reaction similarly to the effect of β-naphthoflavone (Table 11). Phenobarbital had little effect upon the arylhydrocarbon hydroxylase activity *in vitro*.

Discussion--

Monooxygenase activities of untreated rainbow trout hepatic microsomes generally were lower than in many rodent species. For comparison, Table 14 shows representative values from our laboratory for mammalian monooxygenation reactions. Ethoxycoumarin-*O*-deethylation (V_{max}=0.101 nmol/min/mg) and ethoxyresorufin-*O*-deethylation (V_{max}=0.136 nmol/min/mg) in trout were lower than the corresponding mammalian values. However, these values do not reflect a less active hemoprotein P-450, since a 3-4 fold difference in hemoprotein level exists and since, when activity is expressed per unit of hemoprotein, little if any difference exists. Considering the lower concentrations of hemoprotein, trout have significantly higher ethoxyresorufin-*O*-deethylase activity than mammalian species. Similarly, benzo[a]pyrene hydroxylase activity is several-fold higher in trout than in rats (Ahokas et al. 1976, 1977).

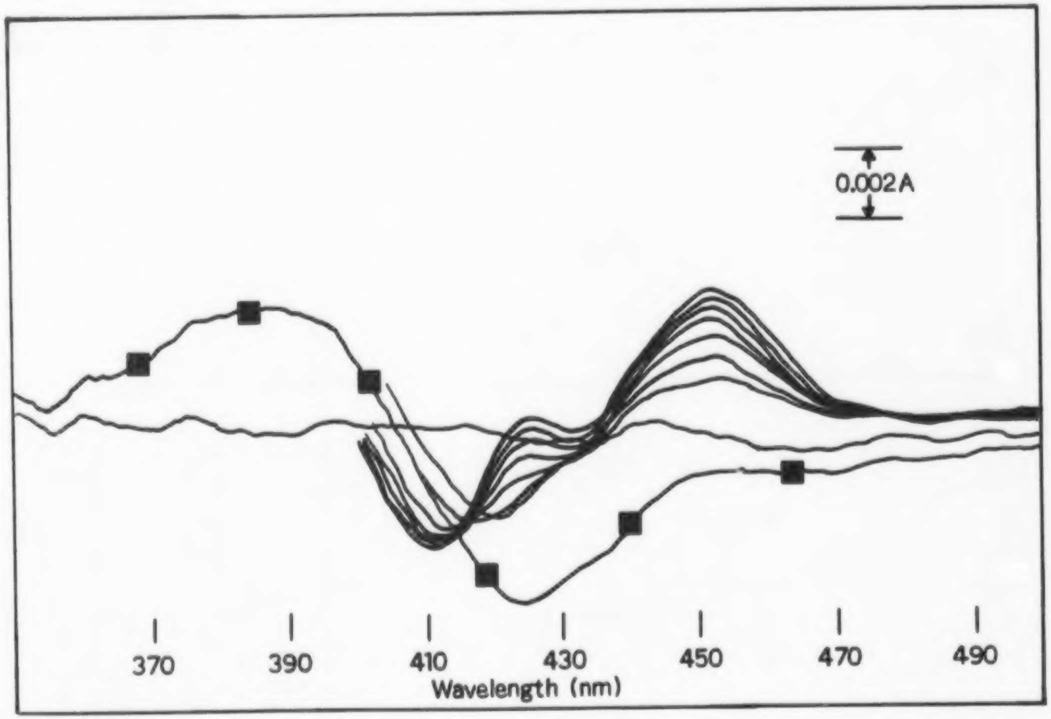

Figure 19. Formation of metabolite-ferrocytochrome P-450 complex in rainbow trout hepatic microsomes. Conditions as described in Figure 6, piperonyl butoxide (■—■) was added to a concentration of 417 μ*M* and the type I binding spectrum was recorded. Then NADPH (250 μ*M*) was added to both cuvettes and the spectrum recorded at 1-min intervals.

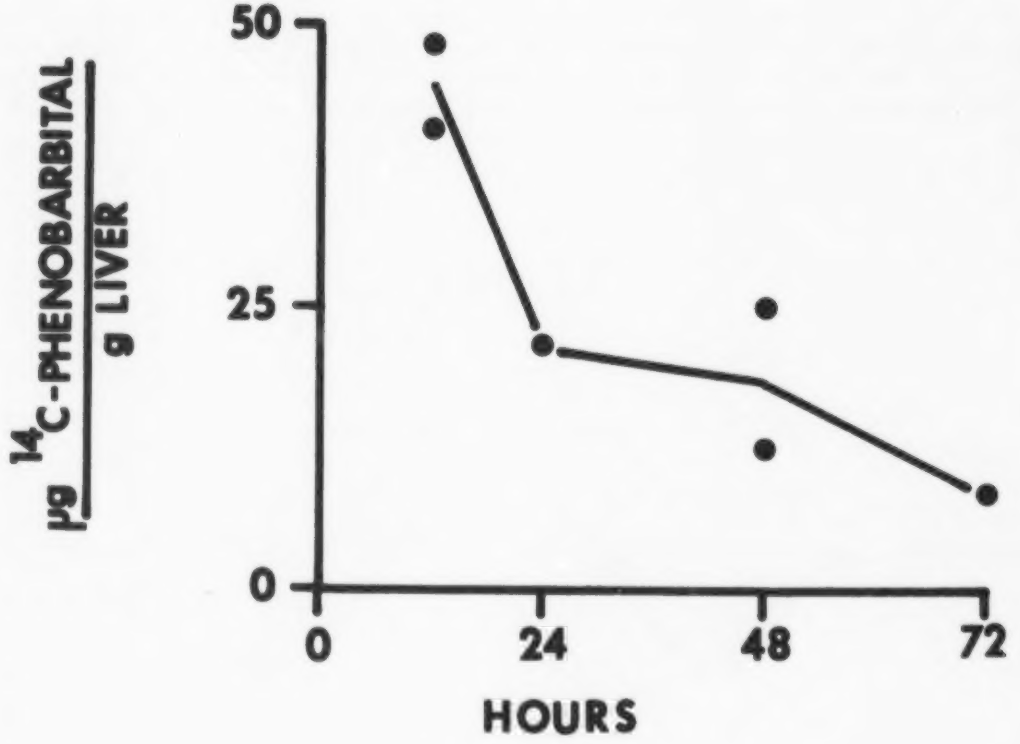

Figure 20. Time course of phenobarbital in trout liver. Rainbow trout (80-100 g) were injected intraperitoneally with 2-^{14}C-phenobarbital (65 mg/kg; 40 μCi/kg) and killed at intervals thereafter. Each point represents amount of ^{14}C-related material in the liver of an individual fish.

TABLE 13. EFFECT OF POTENTIAL INDUCING AGENTS UPON BENZO[a]PYRENE HYDROXYLATION IN CONTROL TROUT HEPATIC MICROSOMES *IN VITRO*

Compound added[a]	Concentration of arylhydrocarbon (benzo[a]pyrene) hydroxylase μ*M*	nmol/min/mg
None[b]	-	0.026 (100)[c]
Aroclor 1242	10	0.025 (96)
	100	0.026 (100)
	500	0.022 (85)
βNF	10	0.005 (19)
	100	0.004 (15)
	500	0.003 (11)
PB	10	0.027 (104)
	100	0.023 (88)
	500	0.021 (81)

[a] Compounds were added to incubation dissolved in 5 μliter *N, N*-dimethyl formamide.
[b] 5 μliter *N, N*-dimethylformamide was added to incubation.
[c] Values in parentheses are percentage of remaining activity.

TABLE 14. TYPICAL HEPATIC MICROSOMAL MONOOXYGENASE ACTIVITIES OF RAT AND MOUSE FOUND IN AUTHORS' LABORATORY

Species and sex	Pretreatment	ECOD[a] nmol/min/mg[c]	EROD[b] nmol/min/mg[c]	Cyt P-450, nmol/mg
Rat, male	None	0.72	0.38	0.71
	3-MC	13.2	64.8	1.54
Rat, female	None	0.31	0.80	0.67
	3-MC	5.65	39.9	1.07
Swiss mouse, male	None	2.00	0.51	0.54
	3-MC	4.61	6.82	1.05

[a] Ethoxycoumarin-*O*-deethylation.
[b] Ethoxyresorufin-*O*-deethylation.
[c] These values represent specific activities (not V_{max}), however these measurements were made at substrate concentrations which gave at least 90% of the V_{max} value.

This study has demonstrated differences in the spectral hepatic hemoprotein properties of rainbow trout when compared to that of lake trout (*Salmo trutta lacustris*) (Ahokas et al. 1976, 1977). Ahokas et al. have indicated that the λ_{max} carboxyferrohemoprotein P-450 of lake trout is at 450.6 nm, whereas in this study the λ_{max} of rainbow trout was consistently at 449 nm. Furthermore, Ahokas et al. could not elicit type I substrate binding spectra in lake trout using hexobarbital or PBO, but both of the compounds elicited type I spectra with microsomes from rainbow trout. Type II spectra found in this study were similar to those observed in rodent species. Also, the trout microsomal hemoprotein P-450 was able to metabolize methylenedioxyphenyl compounds resulting in the formation of metabolic intermediate complexes seen frequently with mammalian hemoprotein P-450 (Hodgson and Philpot 1974).

Several differences between the responses of rainbow trout and rodents to certain xenobiotics are apparent from the present results. Administration of Aroclor 1242 or phenobarbital to several mammalian species results in an increase in the liver:body ratios and the amount of microsomal protein obtained per unit of liver. However, this study has failed to demonstrate such phenomena in rainbow trout with the same compounds.

The hemoprotein P-450 substrates were chosen to cover a broad spectrum of monooxygenase activity. In many rodents, ethylmorphine-*N*-demethylation is characteristic of cytochrome P-450 activity (inducible by phenobarbitals), and benzo[a]pyrene hydroxylase and ethoxycoumarin-*O*-deethylation are catalyzed by both cytochrome P-450 and cytochrome P_1-450, but 7-ethyoxyresorufin-*O*-deethylation apparently can be induced solely by polycyclic aromatic hydrocarbon or mixed-type inducers.

β-Naphthoflavone is representative of a cytochrome P_1-450 inducing agent in rodents (Boobis et al. 1977). This study has demonstrated that it is an effective inducer of enzyme activities associated with cytochrome P_1-450 in rainbow trout. However, only small qualitative alterations in the nature of the constitutive hemoproteins were observed. For example, the Km value for ethoxycoumarin-*O*-deethylation decreased, total hemoprotein P-450 content increased slightly, and the 455:430 ratio of the ethylisocyanide spectra increased slightly. However, unlike the mammalian situation, no blue shift in the Soret maximum of the CO complex of reduced hemoproteins P-450 was observed. Similar observations were made using Aroclor 1242 as an inducing agent.

As found by other investigations (Bend et al. 1973), this study demonstrated phenobarbital to be totally devoid of inducing ability in trout. This inability is probably not a problem of bioavailability since the fish became sedated after injection with phenobarbital and ^{14}C-phenobarbital-related material was found in the liver. Since the ratio of phenobarbital to phenobarbital metabolites was not determined, the lack of induction might be caused by a rapid metabolism of the barbiturate; however, this possibility is unlikely considering the low levels of monooxygenase activity. Furthermore, phenylbutazone and DDT, inducers of the barbiturate class, are also incapable of inducing monooxygenation in fish. This study has also demonstrated a lack of induction of the P-450 associated

N-demethylation of ethylmorphine by Aroclor 1242; this lack contrasts with the mammalian situation, in which Aroclor 1242 induces P -450 and P-450 associated activities.

A lack of induction of monooxygenation by phenobarbital has been reported in the rat fetus (Guenthner and Mannering 1977). However, the simultaneous administration of β-naphthoflavone and phenobarbital resulted in induction of cytochrome P-450 associated activities. A similar experiment carried out in rainbow trout did not result in a synergism (Elcombe and Lech, unpublished observations).

The use of α-Naphthoflavone and metyrapone to inhibit benzo[a]pyrene hydroxylation by trout hepatic microsomes *in vitro* resulted in some interesting observations. α-Naphthoflavone strongly inhibited control and induced AHH activity, but metyrapone had little effect. These results contrast drastically with the mammalian situation, in which β-naphthoflavone stimulates control arylhydrocarbon (benzo[a]pyrene) hydroxylase (cytochrome P-450) but inhibits cytochrome P_1-450 mediated hydroxylation. Metyrapone classically inhibits cytochrome P-450 (control and phenobarbital induced), but has little effect on cytochrome P_1-450. Hence, the results indicate that the control hemoprotein P-450 of rainbow trout is similar to P_1-450. Other indications of this hypothesis are the relatively high control activities of benzo[a]pyrene hydroxylase and ethoxyresorufin-*O*-deethylation and a Soret band for carboxyferrohemoprotein P-450 at 449 nm.

However, the constitutive hemoprotein of trout hepatic microsome is not cytochrome P_1-450. This conclusion is clearly shown by several results: The Soret peak of the carboxyferrocytochrome is at 449 nm, not 448 nm; the EtNC 455:430 peak ratio is 0.28 not 1.0; and a comparison of apoprotein on SDS-PAGE derived from trout and rat cytochrome P-450 demonstrated distinct electrophoretic patterns.

This study also suggests that the physical properties of the induced and control hemoprotein of trout hepatic microsomes are similar. For example, although a 40% increase in total hemoprotein was observed after pretreatment with β-naphthoflavone, no shift in the Soret peak was observed; furthermore, the ratio of the 455:430 peak of the ethyl isocyanide spectra was altered only slightly after induction. Enzymatically, the induced and control hemoproteins have similar qualitative but not quantitative properties. Although induced and control hemoproteins showed similar susceptibilities to inhibition by β-naphthoflavone and metyrapone, increases of 4,081%, 4,455%, and 1,178% in benzo[a]pyrene hydroxylation, ethoxyresorufin-*O*-deethylation, and ethoxycoumarin-*O*-deethylation, respectively, cannot be explained on the basis of a mere 40% increase in the constitutive hemoprotein content of the trout hepatic microsomes. Qualitatively, the only significant alteration was the decrease in the Km for ethoxycoumarin-*O*-deethylation.

The results suggest that different subpopulations of hemoprotein(s) P-450 exist in microsomes for control and β-naphthoflavone treated trout. This hypothesis is supported by the patterns of sodium dodecyl sulfate-polyarylamide gel electropheresis in fish pretreated with β-naphthoflavone

(Figure 21, Figure 22). These patterns exhibited a novel band (which included peroxidase activity) of an apparent molecular weight of 57,000. This band was not observed in microsomes from control fish. The novel band was relatively minor when compared to the total hemoprotein content, and cannot account for the 40% increase in hemoproteins. Hence, β-naphthoflavone and Aroclor 1242 apparently induce constitutive hemoproteins in addition to the novel band at 57,000 daltons. Hence, the novel hemoprotein induced by β-naphthoflavone and Aroclor 1242 apparently exhibits extremely high monooxygenase activity toward benzo[a]pyrene, ethoxyresorufin, and ethoxycoumarin.

STUDIES OF THE FATE OF ORGANIC POLLUTANTS IN FISH

Polycyclic Aromatic Hydrocarbons: Naphthalene and 2-Methylnapthalene

Short-Term Exposures--

The tissue levels of ^{14}C (μg naphthalene + metabolites/g) during an 8-h exposure of fingerling rainbow trout to ^{14}C-naphthalene (0.005 mg/liter) and during a subsequent elimination period are shown in Figure 23 (Melancon and Lech 1978).

Naphthalene was rapidly taken up by the fish, and the levels in the tissues studied were from 22 to 340 times the initial water level of naphthalene after 8 h of exposure. The average biliary concentration (bile volume = 10 μliter) of naphthalene plus metabolites was 3.1 μg/ml (300 times the initial water level of naphthalene) during an 8- to 32-h period. Visceral fat had the highest level of ^{14}C while blood and muscle had the lowest of the tissues studied. The ^{14}C levels in liver, gill, and whole fish were between these extremes.

The results of an exposure at an initial water level of ^{14}C-naphthalene of 0.023 mg/liter are shown in Figure 24. The results of this exposure were similar to those at the lower concentration of naphthalene in terms of the relative levels of ^{14}C in various tissues and in terms of tissue level at 8 h compared to initial water concentration. In this experiment the ratios of tissue ^{14}C to water ^{14}C after 8 h varied from 24 to 585. Bile levels (10 μl of bile/fish) during the 8 to 32 h period averaged 8.2 μg/ml (360 times the initial water level of naphthalene).

The half-lives for the elimination of ^{14}C generally were less than 24 h and were similar for the specific tissues at both exposure levels (Table 15). The exception was fat, for which the half-life was 31 h after the lower-level exposure and 62 h after the higher-level exposure.

In order to evaluate the effect of multiple exposures on elimination, fingerling rainbow trout were exposed to 0.005 mg/liter of ^{14}C-naphthalene for more than on 8-h period with 2-h elimination intervals. The elimination curves on a whole fish basis for double and triple exposures are presented in Figure 25. The half-life values for elimination were 12.3 and 9.8 h for the double exposure and 15.5, 11.3, and 14.7 h for the triple exposure. These values compare well with a half-life of 15.4 h for whole fish for

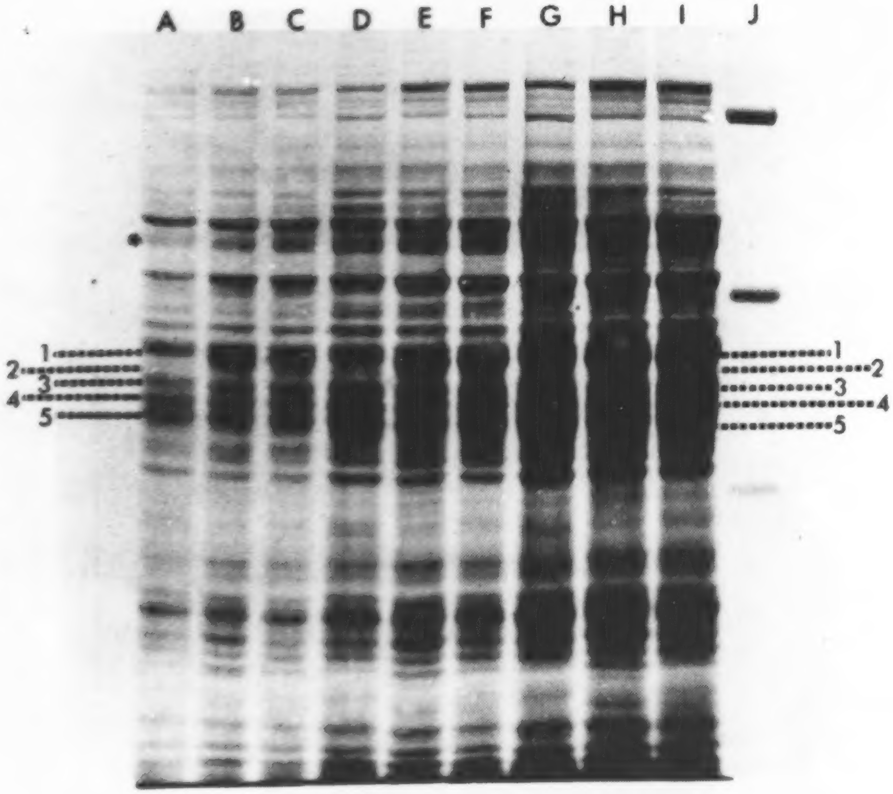

Figure 21. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of microsomes from variously pretreated rainbow trout. Samples A, D, and G; control microsomes at loadings of 45, 90, and 180 μg of protein, respectively. Samples B, E, and H; β-naphthoflavone microsomes (100 mg/kg), loadings as above. Samples C, F, and I; Aroclor 1242-induced microsomes (150 mg/kg), loadings as above. Sample J; standards [*Escherichia coli*, RNA polymerase (β, β′, and β′ subunits), and bovine serum albumin]. Molecular weights: Bands 1, 2, 3, 4, and 5 are 59,500, 57,000, 51,000, 48,000, and 45,000, respectively.

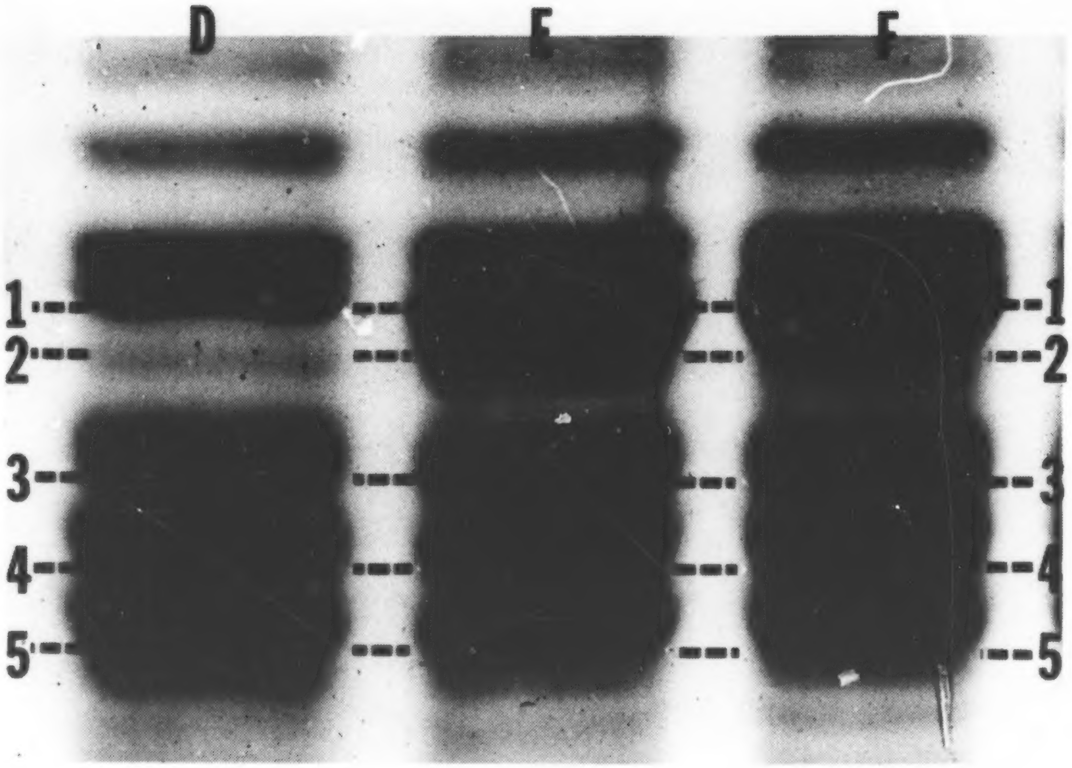

Figure 22. Enlargement of the 40,000- to 60,000-dalton region of samples D, E, and F from Figure 21. Molecular weights for bands 1-5 are as indicated in the legend to Figure 21.

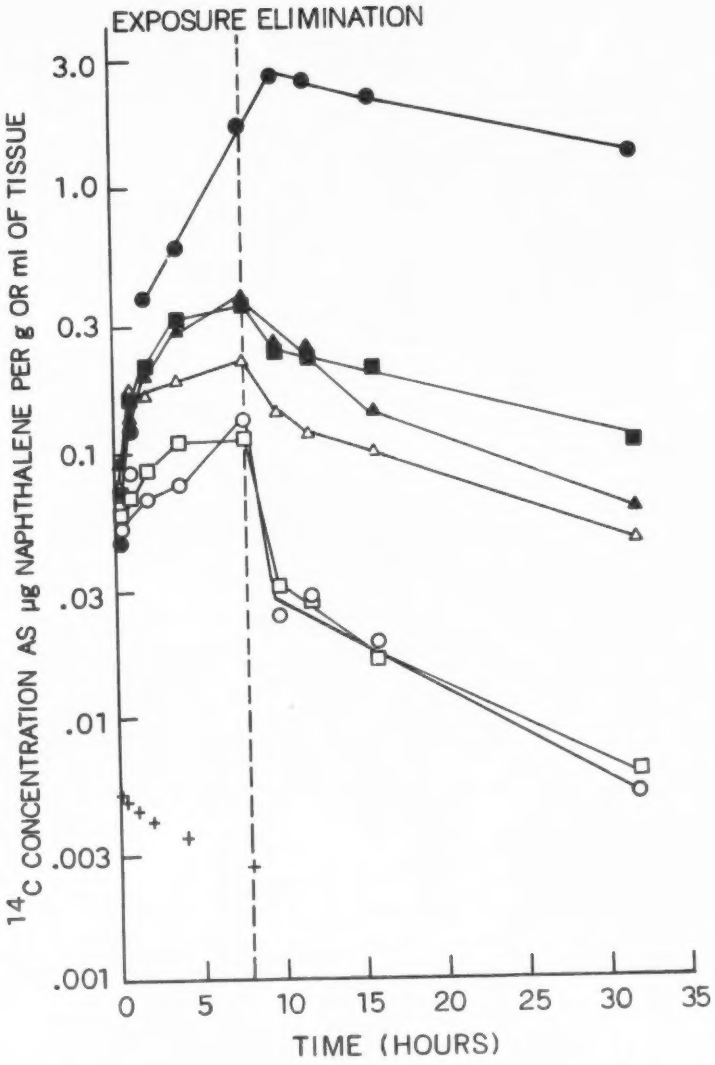

Figure 23. Tissue levels of ^{14}C during exposure to ^{14}C-naphthalene (0.005 mg/liter) and subsequent elimination. Data are the average of values from five trout except for the 8 h values which represent 10 trout. ● = fat, ▲ = whole fish, ■ = liver, △ = gill, ○ = muscle, □ = blood, and + = exposure water.

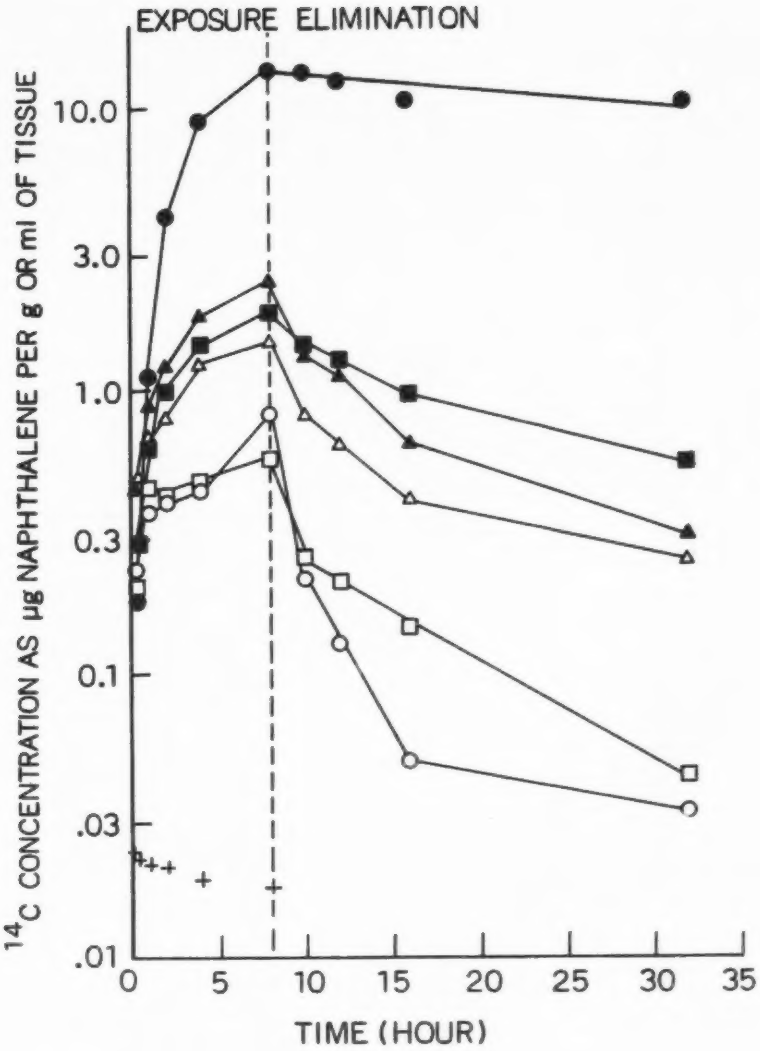

Figure 24. Tissue levels of ^{14}C during exposure to ^{14}C-naphthalene (0.023 mg/liter) and subsequent elimination. Data are the average of values from five trout except for the 8 h values which represent 10 trout. ● = fat, ▲ = whole fish, ■ = liver, △ = gill, ○ = muscle, □ = blood, and + = exposure water.

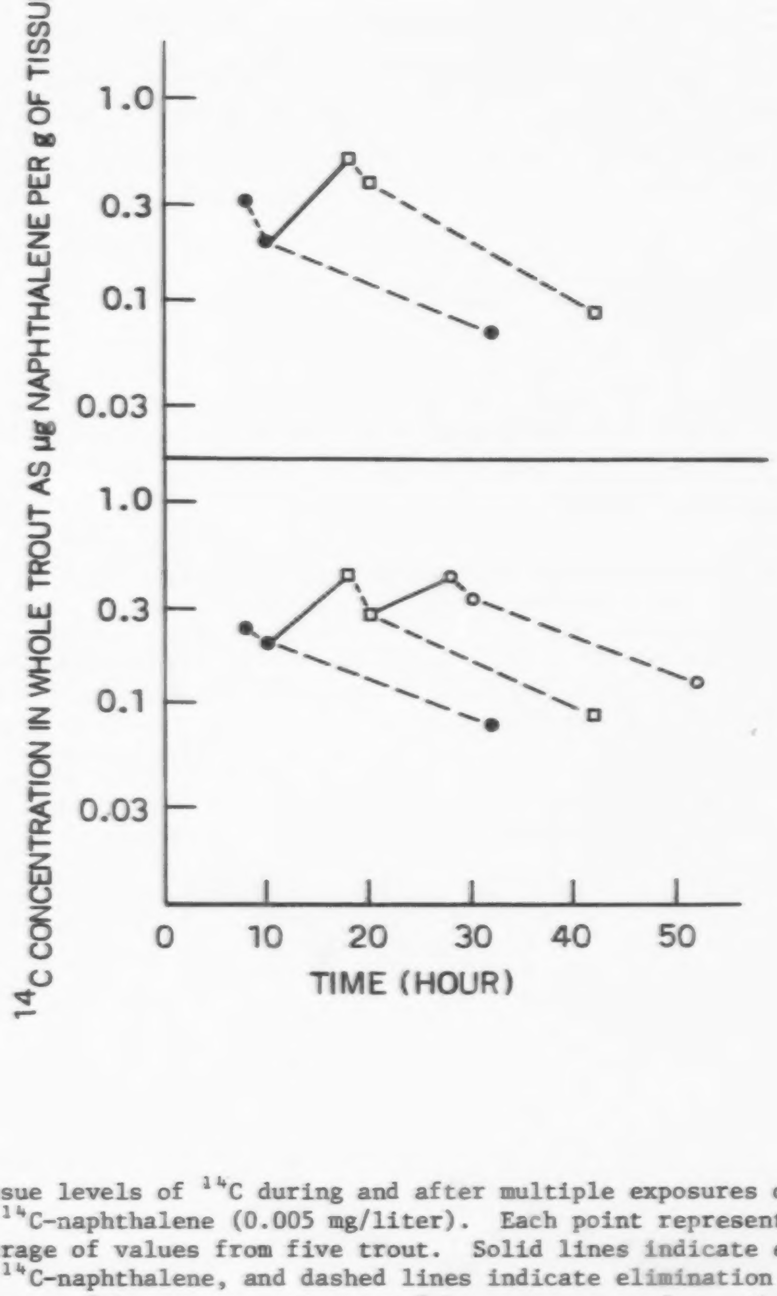

Figure 25. Tissue levels of ^{14}C during and after multiple exposures of trout to ^{14}C-naphthalene (0.005 mg/liter). Each point represents the average of values from five trout. Solid lines indicate exposure to ^{14}C-naphthalene, and dashed lines indicate elimination periods. ● = one 8-h exposure, □ = two 8-h exposures, and ○ = three 8-h exposures.

TABLE 15. ELIMINATION HALF-LIVES OF ^{14}C FROM FINGERLING RAINBOW TROUT EXPOSED TO ^{14}C-NAPHTHALENE IN WATER ON A SHORT-TERM BASIS

Exposure level mg/liter	Exposure duration, h	Elimination duration, h	Half-life of ^{14}C elimination, h					
			Muscle	Blood	Liver	Gill	Fat	Whole fish
0.005	8	24	6.5	7.1	11.9	9.7	31.3	15.4
0.023	8	24	6.5	7.5	11.0	9.0	62.2	14.6

elimination of ^{14}C after a single exposure to 0.005 mg/liter of ^{14}C-naphthalene for 8 h and show no striking differences resulting from the multiple exposures.

Long-Term Exposures--

Statis exposures such as those described above are of limited value because the water concentration of the compounds under study decreased during the exposure period. Therefore, utilizing a continuous flow system, we studied the uptake of ^{14}C-naphthalene (0.023 mg/liter) by fingerling rainbow trout for approximately 4 weeks and the subsequent elimination of accumulated ^{14}C by the fish (Figure 26). As in the short term studies, muscle and blood levels of ^{14}C were similar and were considerably lower than liver concentrations. Muscle and blood levels of naphthalene were 25 times the water concentration of naphthalene and liver levels were 175 times the water level of naphthalene after 16 days of exposure.

A similar experiment was performed to study the uptake and elimination of ^{14}C-methylnaphthalene (Figure 27). In this study, liver again contained the highest levels of the ^{14}C label, while muscle and blood contained lower levels. The concentration of 2-methylnaphthalene in all three tissues had reached a plateau (100 to 300 times the water concentration of 2-methylnaphthalene) long before the end of the exposure period. Muscle levels of 2-methylnaphthalene reached a maximum after 9 days of exposure and showed definite decreases during the remainder of the exposure. After the fish had been in fresh water for 4 days, the radioactivity in 20 µliter of blood was no longer significantly above background. The half-life values for elimination of the two chemicals from the tissues studied are given in Table 16.

The elimination of ^{14}C accumulated from ^{14}C-naphthalene during the long-term exposure took much longer than after the short term exposures. Although blood ^{14}C levels showed an early decrease, all three tissues studied showed a gradual elimination of ^{14}C. The elimination of ^{14}C accumulated from ^{14}C-2-methylnaphthalene during long-term exposure was quite different from that following long-term exposure to ^{14}C-naphthalene. After

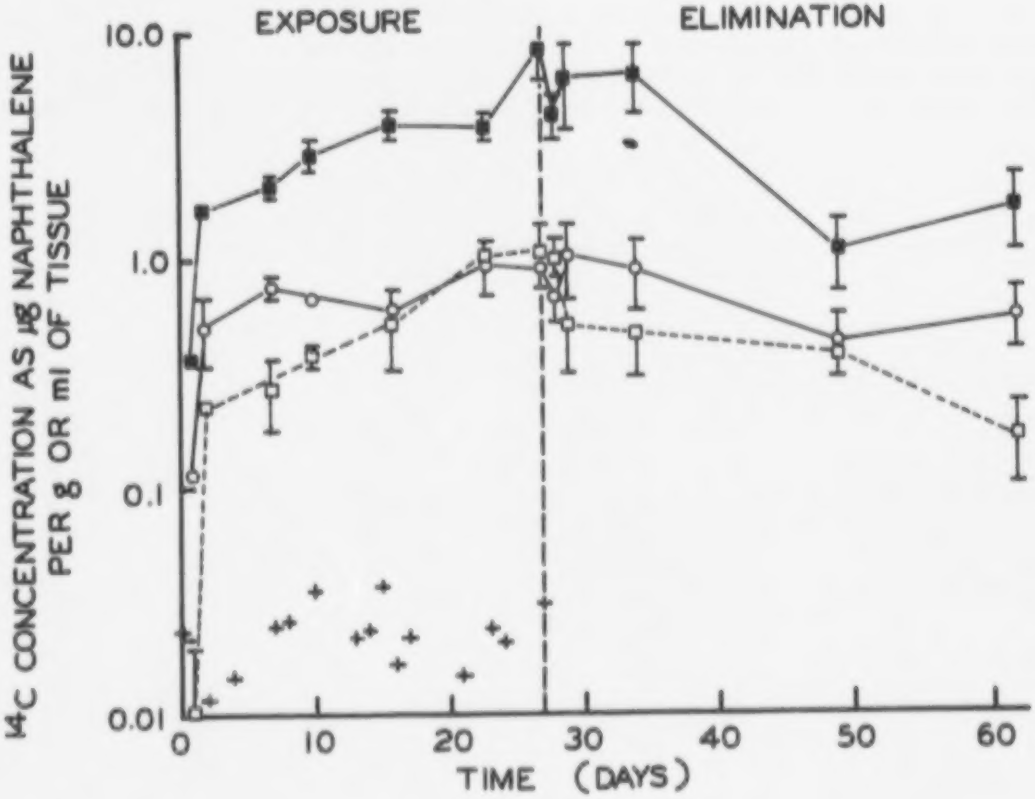

Figure 26. Tissue levels of ^{14}C in trout during a 27-day exposure to ^{14}C-naphthalene and subsequent elimination. Each point represents the average of values from five trout, and the vertical lines represent the standard error. The average concentration of ^{14}C-naphthalene in water during the exposure was 0.023 mg/liter. ■ = liver, ○ = muscle, □ = blood, and + = exposure water.

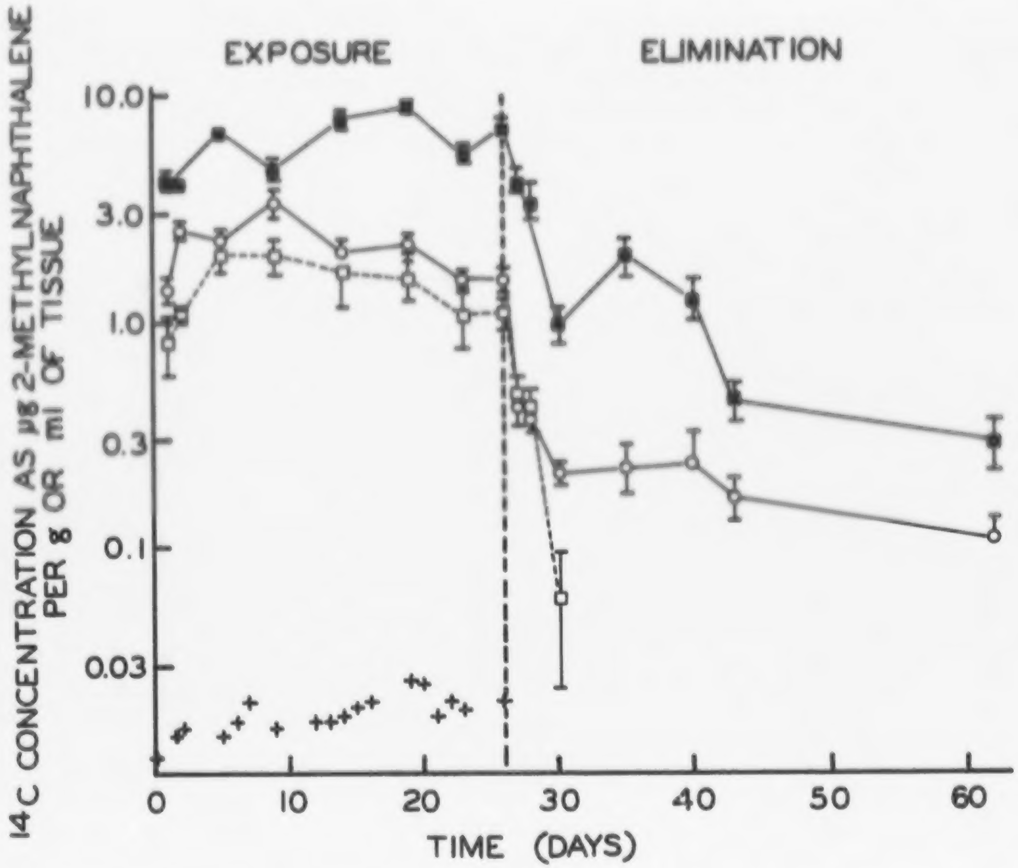

Figure 27. Tissue levels of ^{14}C in trout during a 26-day exposure to ^{14}C-2-methylnaphthalene and subsequent elimination. Each point represents the average value from five trout, and the vertical lines represent the standard error. The average concentration of ^{14}C-2-methylnaphthalene in water during the exposure was 0.017 mg/liter. ■ = liver, ○ = muscle, □ = blood, and + = exposure water.

TABLE 16. ELIMINATION HALF-LIVES OF ^{14}C FROM FISH EXPOSED TO AQUEOUS ^{14}C-NAPHTHALENE OR ^{14}C-2-METHYLNAPHTHALENE FOR SEVERAL WEEKS

Exposure	Half-lives, h Muscle	Liver	Blood	Whole fish
Trout Naphthalene 0.017 mg/liter	909	343	379	
Trout 2-Methylnaphthalene 0.023 mg/liter	13[a] 711	211	23	
Carp 2-Methylnaphthalene 0.013 mg/liter	31[b] 1942	59[b] 781		
Bluegill sunfish 2-Methylnaphthalene 0.013 mg/liter				< 24[c] 353

[a] When two values are given, the upper value is the early rapid phase of elimination and the lower value is the latter slower phase. The slope and intercept for the slow phase of elimination were calculated using the data from days 4-36. The data from days 0-2 were corrected for this, and the resulting values were used to calculate slope for the rapid phase of elimination from days 0-2.

[b] Data for flow phase, days 8-73, and for rapid phase, days 0-3.

[c] Data for slow phase, days 1-26, about 60% of ^{14}C eliminated during first day.

exposure to ^{14}C-2-methylnaphthalene, ^{14}C levels in blood dropped rapidly and an initial rapid drop in ^{14}C levels in muscle was observed followed by a gradual loss comparable to that observed with naphthalene. Because of the fluctuations of the ^{14}C levels in liver, the elimination from liver was calculated as a single-phase elimination and resulted in a half-life similar to that observed with naphthalene.

Levels of ^{14}C in bile increased during the early part of the exposure, reaching a maximum at 2-3 weeks for each compound. After this period, ^{14}C

reaching a maximum at 2-3 weeks for each compound. After this period, ^{14}C

levels in bile dropped more rapidly in trout exposed to ^{14}C-naphthalene than in those exposed to ^{14}C-2-methylnaphthalene. At 24 h naphthalene and metabolites in bile totaled 12 μg/ml (approximately 500 times the water concentration of naphthalene), and 2-methylnaphthalene and metabolites totaled 28 μg/ml (approximately 1,600 times the water concentration of

2-methylnaphthalene). The maximum for naphthalene and metabolites was 300 µg/ml (approximately 13,000 times the water concentration of naphthalene) while that for 2-methylnaphthalene and metabolites was 400 µg/ml (approximately 23,500 times the water concentration of 2-methylnaphthalene). Initially greater concentrations of ^{14}C in bile resulting from exposure to ^{14}C-2-methylnaphthalene compared with exposure to ^{14}C-naphthalene were probably related to the greater uptake of 2-methylnaphthalene. Later in the exposure and during the first week of elimination, however, the differences were much more striking and may be related to the considerable loss of ^{14}C from muscle of trout exposed to ^{14}C-2-methylnaphahtlene at these times.

Small carp and bluegill sunfish were exposed similarly to ^{14}C-2-methylnaphthalene (Table 17 and Table 18) (Melancon and Lech, 1979). Because of the very small size of the latter, ^{14}C levels were determined only for the whole fish. The elimination of ^{14}C from these species was similar to that observed with trout. A substantial loss of ^{14}C from tissue occurred during the first 24 h of elimination followed by a much more gradual loss. The half-lives for elimination of ^{14}C from the various tissues in these experiments are listed in Table 16.

While biliary ^{14}C in the trout reached a maximum of 10,000 to 20,000 times the water level of ^{14}C with both compounds, biliary ^{14}C in the carp was over 100,000 times the water level of ^{14}C on several occasions. Interestingly, these high levels of biliary ^{14}C continued well into the elimination period after exposure of trout and carp to ^{14}C-2-methylnaphthalene. The levels of biliary ^{14}C were much however, during the elimination period following exposure of trout to ^{14}C-naphthalene.

The biliary levels of ^{14}C in carp exposed to ^{14}C-2-methylnaphthalene were particularly interesting. We have suggested (Statham et al. 1976) the use of fish bile in the monitoring of aquatic pollution. The results support this suggestion since bile contained ^{14}C at over 100,000 times the level of ^{14}C in the exposure water and these concentrations persisted for several days after the exposure was terminated.

Metabolism Studies--

The ^{14}C present in bile and in acetone extracts of muscle and liver from trout exposed to 0.5 mg/liter of ^{14}C-naphthalene and ^{14}C-2-methylnaphthalene was examined for the presence of metabolites (polar ^{14}C-labeled material) by thin-layer chromatography (Figure 28). With CCl_4 as the solvent, the unchanged hydrocarbons had Rf-values of approximately 0.5, while metabolites remained at the origin. Because of the volatility of the unchanged hydrocarbons, much of the ^{14}C was lost during TLC, but the percent of the spotted amount of ^{14}C which remained at the origin gave some degree of quantification of the amount of metabolites present. For both hydrocarbons bile contained at least 65-70% metabolites, liver 5-10% metabolites, and muscle < 1% metabolites. More recent values of percent of metabolites in bile derived from hexane:water partitioning are generally over 85% and frequently over 95%.

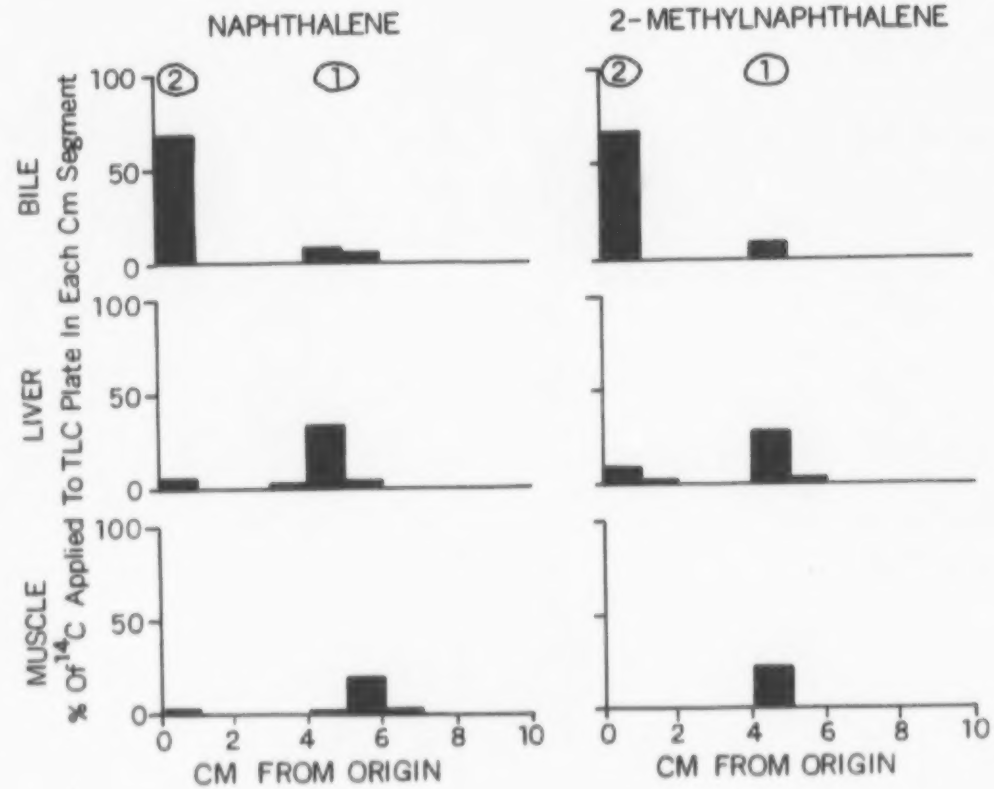

Figure 28. TLC profiles of tissue radioactivity from rainbow trout exposed to 0.5 mg/liter ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene for 24 h. Samples of bile or of acetone tissue extracts were chromatographed with CCl_4 as solvent. The radioactivity present in each 1-cm segment is expressed as % of total radioactivity applied to the TLC plate. The circled number 1 represents the mobility of the appropriate parent compound, and the circled number 2 represents that of the appropriate monohydroxylated compound.

TABLE 17. UPTAKE AND ELIMINATION OF ^{14}C-METHYLNAPHTHALENE BY CARP AFTER EXPOSURE TO 0.013 MG ^{14}C-2-METHYLNAPHTHALENE/LITER

	Time, days	^{14}C-2-methylnaphthalene and metabolites µg/g of tissue	
		Muscle	Liver
Exposure	1	0.67 ± 0.02	5.82 ± 0.75
	4	1.47 ± 0.28	7.55 ± 1.06
	8	1.31 ± 0.09	6.89 ± 0.76
	15	1.42 ± 0.20	8.31 ± 0.82
	22	1.13 ± 0.08	10.19 ± 0.49
	26	1.60 ± 0.17	12.82 ± 1.73
Elimination	1	0.96 ± 0.08	7.20 ± 1.03
	2	1.01 ± 0.08	5.81 ± 0.48
	3	0.62 ± 0.14	6.13 ± 1.17
	8	0.44 ± 0.07	2.76 ± 0.45
	19	0.26 ± 0.04	1.56 ± 0.63
	26	0.41 ± 0.09	2.05 ± 0.42
	32	0.45 ± 0.11	1.88 ± 0.29
	73	0.22 ± 0.08	0.63 ± 0.09

TABLE 18. UPTAKE AND ELIMINATION OF ^{14}C-2-METHYLNAPHTHALENE BY BLUEGILL SUNFISH AFTER EXPOSURE TO 0.013 MG ^{14}C-2-METHYLNAPHTHALENE/LITER

	Time, days	^{14}C-2-methylnaphthalene and metabolites µg/g of tissues
		Whole fish
Exposure	1	2.17 ± 0.35
	4	2.94 ± 1.14
	8	5.41 ± 1.65
	15	3.68 ± 0.83
	22	6.30 ± 2.29
	26	5.24 ± 1.36
Elimination	1	2.16 ± 0.29
	2	2.25 ± 0.52
	3	1.62 ± 0.26
	8	1.86 ± 0.41
	19	1.14 ± 0.40
	26	0.58 ± 0.09

Additional TLC in other solvents demonstrated that most of the metabolites in bile were more polar than the hydroxyl derivatives of naphthalene and 2-methylnaphthalene and were probably present as conjugates. Investigations are continuing on the isolation and identification of these metabolites, and the results of these studies will be reported at a later time.

The data for the elimination of ^{14}C-naphthalene following short-term and long-term exposures indicated that elimination of ^{14}C after the long-term exposure was much slower than after short-term exposures. Although no information of short-term exposure and elimination using ^{14}C-2-methylnaphthalene has been presented, some limited data indicate that the half-lives for elimination of this compound from several tissues following 8-h exposures were considerably < 24 h. Elimination of ^{14}C from muscle and blood after long-term exposure to ^{14}C-2-methylnaphthalene had a rapid phase with half-lives not greatly different from those following short-term exposure to ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene. A much slower elimination phase also occurred, which was comparable to the elimination seen following long-term exposure to ^{14}C-naphthalene.

A possible explanation for the slower elimination of ^{14}C following long-term exposure to these compounds as compared to that following short-term exposures was suggested by two recent reports. Roubal et al. (1977) found that metabolites of naphthalene are present in tissues of coho salmon fingerlings following intraperitoneal injection of ^{14}C-naphthalene, and Sanborn and Malins (1977) reported that naphthalene is released more rapidly from shrimp larvae than are naphthalene metabolites.

Although in this study TLC of the muscle extracts from trout exposed to 0.5 mg/liter ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene for 24 h showed that < 1% of the ^{14}C was present as metabolites, the possibility existed that the longer term continuous-flow studies at lower levels might have resulted in greater percentages of metabolites in muscle. Despite the relatively low levels of ^{14}C occurring in muscle samples at certain stages of the long term study, estimation of the percent of metabolites present in muscle seemed important. Therefore, muscle filets from fingerling rainbow trout, which had been exposed to ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene for either 1 day, 4 weeks, or 4 weeks with a subsequent elimination period, were extracted, and the ^{14}C present in these extracts was examined by TLC. The data in Table 19 show that the proportions of ^{14}C in muscle present as polar material were greater after a week of elimination than during the exposure period for both compounds. In the case of 2-methylnaphthalene, the difference in percent of metabolites is striking. Only 1% of ^{14}C in muscle was present as metabolites during the exposure and at the beginning of the elimination period; however, 24% of ^{14}C in muscle was present as metabolites during the slow phase of elimination. No striking change occurred in the percent of metabolites present in the muscle from fish exposed to ^{14}C-naphthalene.

Following long-term exposure of trout to ^{14}C-2-methylnaphthalene the elimination of ^{14}C from muscle initially was very rapid at a time when the ^{14}C was present mostly as 2-methylnaphthalene. Later, the elimination was slower when a substantial fraction of the ^{14}C represented metabolites of 2-methylnaphthalene. These results are consistent with differential rates of elimination of 2-methylnaphthalene and its metabolites from muscle. During exposure of trout to naphthalene a significant fraction of the ^{14}C in muscle was present as metabolites early in the exposure period. Although this fraction increased after a week of elimination, the elimination rate of ^{14}C was monophasic at a rate similar to the slow phase of elimination of ^{14}C from the muscle of trout exposed to 2-methylnaphthalene.

TABLE 19. FRACTION OF ^{14}C IN MUSCLE FROM TROUT EXPOSED TO ^{14}C-NAPHTHALENE OR ^{14}C-2-METHYLNAPHTHALENE PRESENT AS POLAR COMPOUNDS

Exposure	^{14}C as polar compounds,[a] %
Naphthalene	
1-day exposure	21
27-day exposure	12
27-day exposure plus 9-day elimination	34
2-Methylnaphthalene	
1-day exposure	1.5
26-day exposure	1.1
26-day exposure plus 7-day elimination	24

[a] Values shown are derived from muscle samples analyzed at the end of the indicated experimental periods

Whether these metabolites are formed in muscle or in another tissue such as liver is not known. During long-term exposures bile was found to contain ^{14}C at thousands of times the water exposure levels. However, these biliary metabolites were not necessarily excreted in the feces and the possibility exists of enterohepatic circulation with subsequent accumulation of these metabolites in such tissues as muscle. Regardless of how these metabolites accumulate in muscle, the possibility of such accumulation during relatively long-term exposures of fish to chemicals must be considered when experiments are designed to evaluate such factors as bioaccumulation and equilibrium concentrations of various chemicals in fish.

Compared to naphthalene, 2-methylnapthalene is taken up more rapidly by rainbow trout, intitally is accumulated to higher levels in all tissues studied, is stored in muscle to a much greater extent as the parent compound and is eliminated more rapidly from the muscle, liver and blood. Data also suggest that over longer periods of time, exposure of trout to naphthalene might lead to higher tissue leels of the parent compound plus metabolites than would be the case with 2-methylnaphthalene. Although long-term exposures are often difficult to interpret because of possible enterohepatic cycling of metabolites within the fish or cycling between fish, water, and flora, the results reported herein indicate that short-term exposures alone could be misleading for predicting long-term tissue levels of certain chemicals.

A summary of the biliary levels of ^{14}C resulting from the exposure of trout to ^{14}C-naphthalene and trout, carp, and sheepshead to ^{14}C-2-methylnaphthalene is presented in Table 20. In general, exposure to ^{14}C-2-methylnaphthalene resulted in higher biliary levels of ^{14}C than did exposure to ^{14}C naphthalene. Biliary levels of ^{14}C in fish exposed to ^{14}C-2-methylnaphthalene were higher for carp than for trout or sheepshead.

The differences in biliary levels of ^{14}C in trout exposed to these chemicals may be explained in part by the greater tissue levels of ^{14}C resuting from exposure to ^{14}C-2-methylnaphthalene. Biliary levels of ^{14}C during the elimination periods following 4 weeks of exposure to these chemicals did not substantiate this explanation. During the elimination periods the biliary levels of ^{14}C in fish exposed to ^{14}C-2-methylnaphthalene were much higher than those in fish exposed to ^{14}C-naphthalene at times when tissue levels of ^{14}C in the methylnaphthalene-exposed trout had dropped below those of the napththalene-exposed trout.

When quantities of bile from the long-term exposures of trout to ^{14}C-naphthalene or 2-methylnapthalene were great enough, pooled bile from each sampling time was examined by hexane:water partitioning. In general, over 98% of the ^{14}C in the bile represented metabolites, and over 90% of the ^{14}C represented very polar metabolites, probably conjugated metabolites.

The TLC of bile from small carp exposed to ^{14}C-2-methylnaphthalene showed that the peaks of ^{14}C were much more polar than 2-methylnaphthalene and 2-methyl-1-naphthol (Figure 29). The standards, naphthalene sulfate, naphthalene glucopyranoside, and naphthalene glucoronide did not co-chromatograph with either of the two major radioactive areas resulting from TLC in the butanol:NH_4OH:water solvent system. TLC of the biliary ^{14}C from similarly exposed larger carp with the same solvent system showed a similar distribution of radioactivity (Figure 30). While biliary ^{14}C from sheepshead exposed to ^{14}C-2-methylnaphthalene also resulted in two radioactivity peaks in this solvent system, the peaks different from those present in carp. As shown later, biliary ^{14}C in trout appears to be intermediate between these two species (Figure 31).

Chlorinated Hydrocarbons

The uptake, metabolism, disposition, and elimination of several chlorinated benzenes also were studied. The first two, pentachlorophenol (PCP) and pentachloroanisole (PCA), which differ only by a methyl group, were studied at the same time and are discussed together. The third--

TABLE 20. BILIARY ^{14}C FOLLOWING EXPOSURE OF FISH TO ^{14}C-NAPHTHALENE OR ^{14}C-2-METHYLNAPHTHALENE

Compound	Species	Average fish weight, g	Inital water level, mg/liter	Exposure duration	Biliary level--parent compound and metabolites, mg/ml	Biliary level: water level
Naphthalene	Trout	11	0.005	24 h	0.002	370
	Trout	6	0.005	8 h[a]	0.002	384
	Trout	7	0.023	8 h[a]	0.014	591
	Trout	5	0.017	16 days	0.327	19,200
	Trout	185	0.467	24 h	0.033	71
	Trout	84	0.457	24 h	0.025	55
2-methylnaphthalene	Trout	11	0.005	24 h	0.013	2,600
	Trout	4	0.023	14 days	0.434	18,900
	Trout	74	0.494	24 h	0.107	217
	Trout	92	0.430	24 h	0.115	267
	Carp	4	0.013	8 days	1.835	141,200
	Carp	25	0.337	24 h	0.654	1,940
	Carp	1,050	0.473	24 h	0.691	1,460
	Sheepshead	450-550	0.072	48 h	0.026	361

[a] Exposure of 8 h plus 24 h in fresh water.

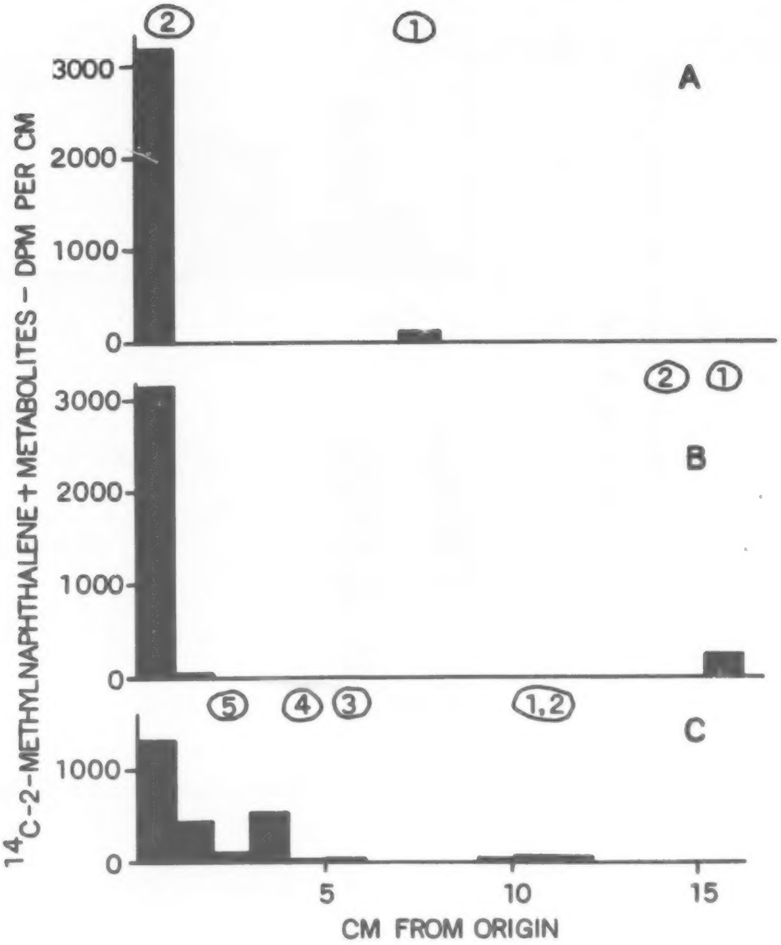

Figure 29. Thin-layer chromatography of biliary ^{14}C from carp exposed to 0.337 mg of ^{14}C-2-methylnaphthalene/liter for 24 h. The solvents used were A = CCL , B = benzene:acetone (5:1) and C = the organic phase of butanol:NH_4OH:water (4:1:5). The circled numbers represent the mobility of standards where 1 = 2-methyl-naphthalene, 2 = 2-methyl-1-naphthol, 3 = 1-naphthylsulfate, 4 = 1-naphthylglucopyranoside and 5 = 1-naphthylglucuronide.

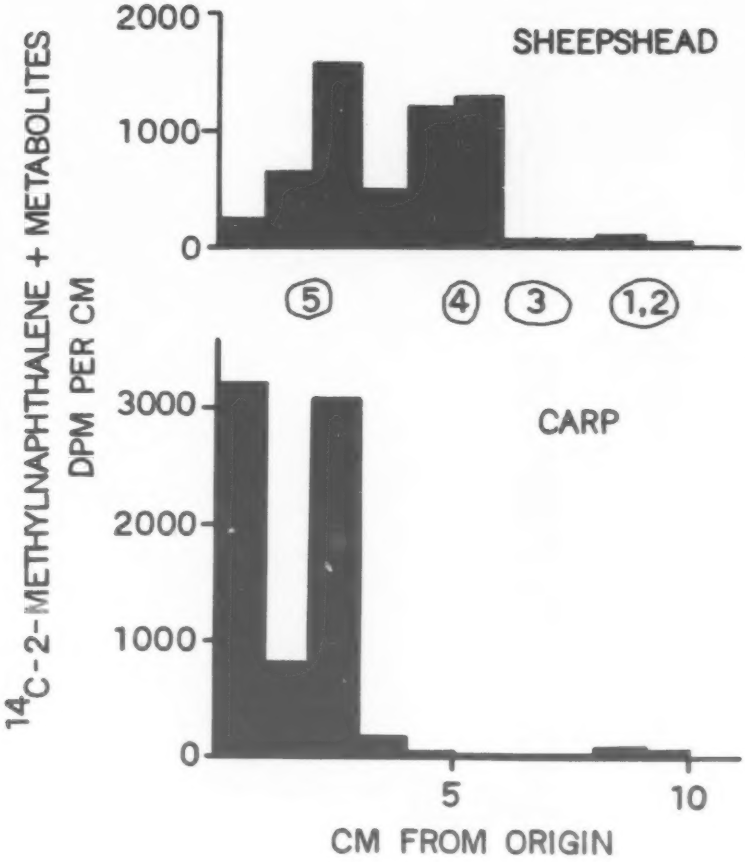

Figure 30. TLC of biliary ^{14}C from carp and sheepshead exposed to ^{14}C-2-methylnaphthalene. The carp were exposed to an initial level of 0.47 mg/liter for 24 h, and the sheepshead were exposed to an initial level of 0.072 mg/liter for 48 h. The solvent used was the organic phase of butanol:NH OH:water (4:1:5). The circled numbers represent the mobility of standards 1 = 2-methylnaphthalene, 2 = 2-methyl-1-naphthol, 3 = 1-naphthylsulfate, 4 = 1-naphthylglucopyranoside and 5 = 1-naphthylglucuronide.

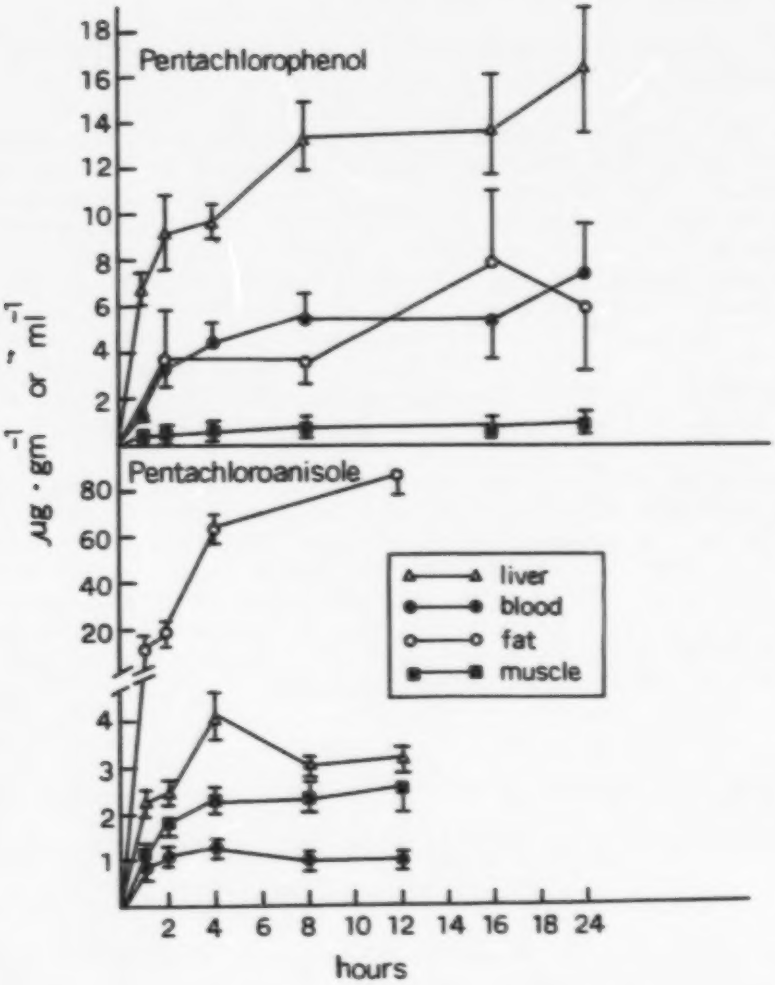

Figure 31. Time course of PCP and PCA in several tissues of rainbow trout. Data are calculated as micrograms of PCP or PCA per gram wet weight. Each point represents the mean ± S.E. from at least six fish in two separate uptake studies.

trichlorobenzene--is discussed separately.

Pentachlorophenol and Pentachloroanisole--

Small rainbow trout were exposed to ^{14}C-PCP or ^{14}C-PCA as described in the methodology section.

The data shown in Figure 31 illustrate that both PCP (upper panel) and PCA (lower panel) are taken up rapidly from water by rainbow trout. Since these static exposures were intended to determine the time course of uptake prior to elimination studies, the plateau of the tissue concentrations should not be taken as steady tissue levels. The plateau in the tissues shown is more likely because of a rapid removal of PCP and PCA from the exposure water rather than a true steady state due to saturation of tissues. Clearly, ^{14}C from PCA was concentrated rapidly in adipose tissue, reaching a tissue ^{14}C to initial water ^{14}C ratio of approximatley 4,000 while the ratio for PCP was approximately 400. On the other hand, PCP appeared to reach higher concentrations in liver than PCA.

Elimination of ^{14}C from tissues of PCA- and PCP-exposed rainbow trout is shown in Figure 32. Although some redistribution of PCP in the muscle and fat during the washout period occurred, the time course of elimination clearly shows that PCA is retained for a much longer period of time in rainbow trout than is PCP. The half-lives for PCP and PCA calculated from these curves are shown in Table 21, and the magnitude of the difference in retention of the compounds is reflected in the extremes in the half-lives for PCP (hours) when compared to PCA (days).

TABLE 21. HALF-LIFE OF PCP AND PCA IN RAINBOW TROUT TISSUES[a]

		Half-lives				
Chemical	Blood	Liver	Fat	Muscle	Gills	Heart
PCP	6.2 h	9.8 h	23.7 h	6.9 h	10.3 h	6.9 h
PCA	6.3 d	6.9 d	23.4 d	6.3 d	--	--

[a] Data calculated from elimination studies shown in Figure 30.

Figure 33 shows the concentrations of ^{14}C, calculated as PCA or PCP, in water, bile, blood, and fat of trout exposed to ^{14}C-PCP (upper) and ^{14}C-PCA (lower). At both sampling times shown, the PCP concentration in bile was much higher than that in fat in the PCP-exposed trout, while the opposite was true with the PCA exposure. The water concentration shown is for reference only and is the value from samples taken at zero time.

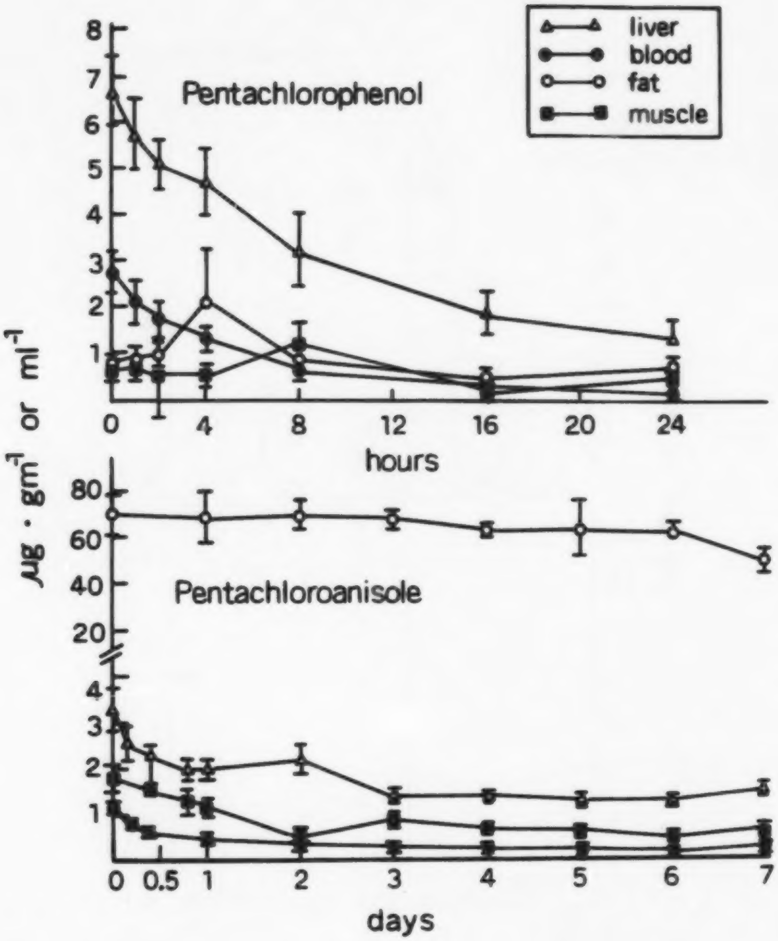

Figure 32. Elimination of ^{14}C from PCP- and PCA-exposed rainbow trout. After a loading exposure the fish were transferred to fresh running water and sampled at the indicated times. Note the difference between time scales in the upper and lower panels. Each part represents the mean ± S.E. from six fish in two separate experiments.

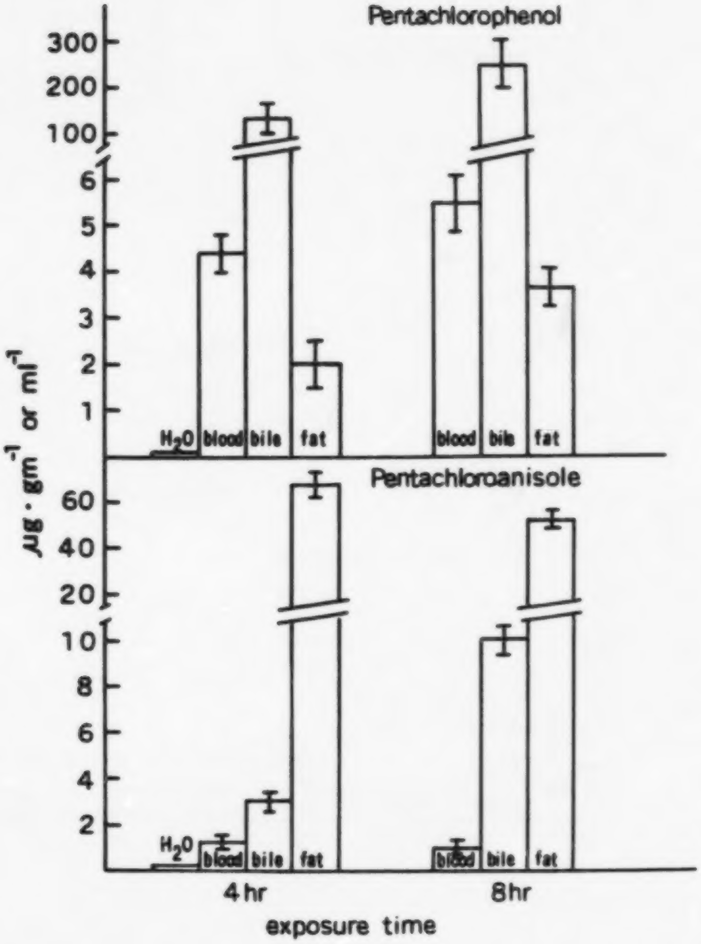

Figure 33. ^{14}C in blood, bile, and fat of rainbow trout exposed to ^{14}C PCP and ^{14}C PCA for 4 and 8 h. The height of the bars represents the mean ± S.E. from at least six fish in two separate exposures. The water concentration is for zero time and is presented as a reference point only.

Thin-layer chromatographic analysis of acetone extracts of the muscle, liver, and bile from PCP-exposed trout is shown in Figure 34. The data clearly indicate the absence of detectable PCA in any of the tissue samples analyzed from PCP-exposed fish. The polar material seen at the origin in the bile and liver appear to be PCP-glucuronide, since treatment of the bile with β-glucuronidase decreased this peak and increased the radioactivity in the area corresponding to the authentic PCP standard. A third radioactive peak can be seen with an R_f-value slightly less than that of PCP, but this material was not identified. The results of gas chromatography/mass spectroscopy (GC/MS) analysis of partially purified (by TLC) extracts of the muscle, liver, and hydrolyzed bile from PCP-exposed trout are shown Table 22. Before chemical methylation of the extracts, the only PCP-related compound found was unchanged PCP. After methylation the latter material afforded the identical retention time and MS of PCA. No GC/MS evidence existed for PCA, tetrachlorohydroquinone, or tetrachloroquinone in the tissues examined. Although similar studies of these tissues from the PCA-exposed trout revealed only PCA in muscle and a trace of more polar material in liver (Figure 35), bile appeared to contain a polar metabolite of PCA. Treatment of the bile with β-glucuronidase (lower panel) resulted in the appearance of a new radioactive peak corresponding to the mobility of the PCP standard.

Since the appearance of conjugated PCP in the bile of PCA-exposed trout suggested demethylation of PCA, the effect of PBO--an inhibitor of microsomal mixed-function oxidases--on the biliary metabolite pattern of

TABLE 22. THE GC/MS AND TLC ANALYSIS OF TISSUE EXTRACTS FROM RAINBOW TROUT EXPOSED TO ^{14}C-PCP[a]

Sample	Molecular ion (M+) observed	^{14}C-R_f value
PCP standard	264, 266, 268, 270	0.46
PCA standard	278, 280, 282, 284	0.85
Liver extract	264, 266, 268, 270	0.46
Methylated liver extract	278, 280, 282, 284	0.85
Muscle extract	264, 266, 268, 270	0.46
Methylated muscle extract	278, 280, 282, 284	0.85
Bile extract[b]	264, 266, 268, 270	0.46
Methylated bile extract	278, 280, 282, 284	0.85

[a] See Glickman et al. (1977) for experimental details.

[b] Hydrolysis by β-glucuronidase caused residue at R_f of zero to migrate to an R_f of 0.46.

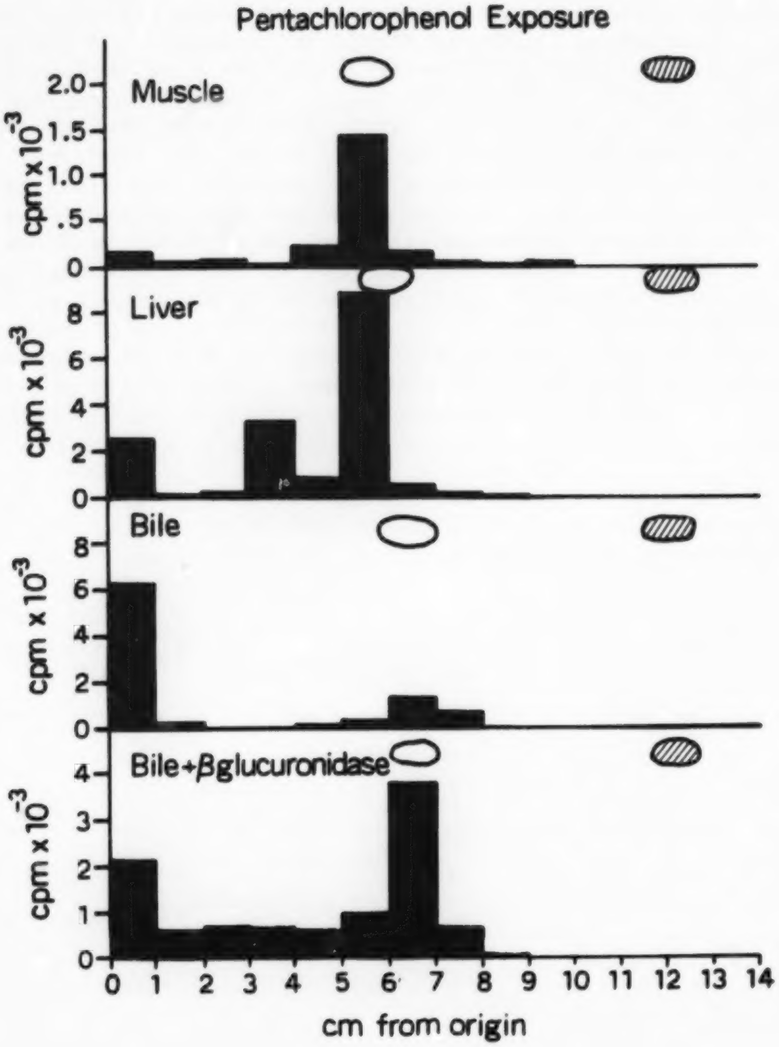

Figure 34. Thin-layer radiochromatogram of samples prepared from tissues of rainbow trout exposed to ^{14}C-PCP. Clear spot is authentic PCP; hatched spot is authentic PCA.

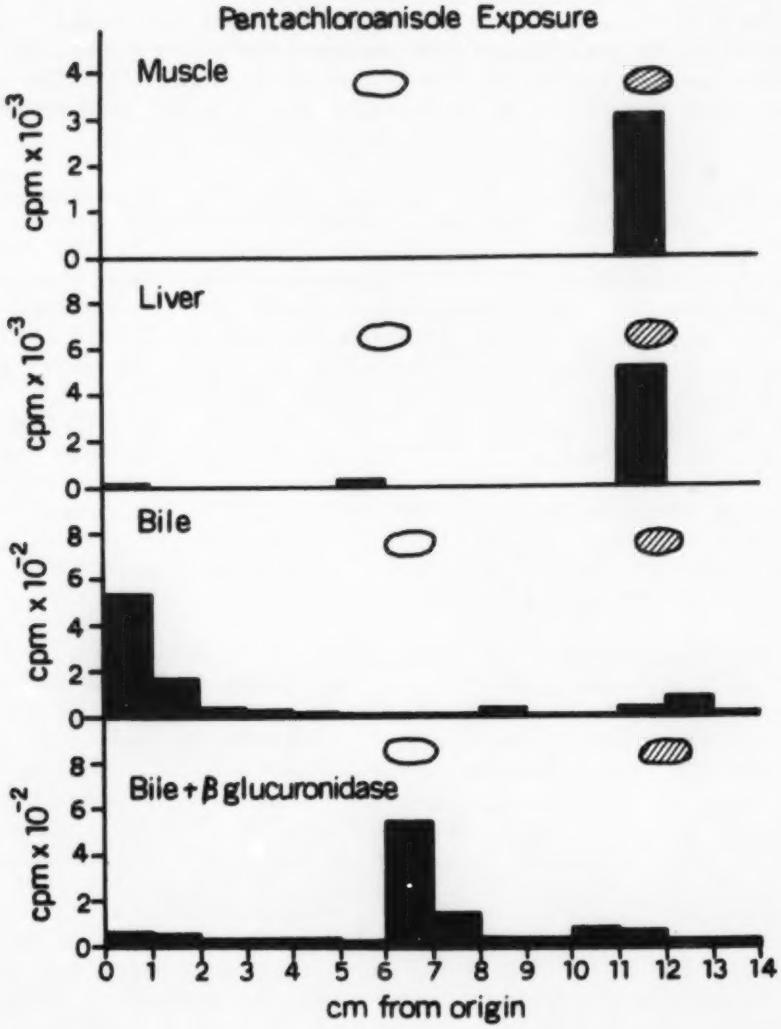
Pentachloroanisole Exposure

Figure 35. Thin layer-radiochromatogram of samples prepared from tissues of rainbow trout exposed to ^{14}C-PCA. Clear spot is authentic PCP; hatched spot is authentic PCA.

PCA-exposed trout was studied. The data in Table 23 show that inclusion of 1 mg/liter of PBO in the tank water reduced the bile content of ^{14}C. Calculation of the distribution of radioactivity in bile--based on the TLC analysis of the bile from the control and PBO groups--indicated that the concentration of PCP-glucuronide was reduced in bile in the PBO-treated fish, and the concentration of unconjugated-PCA was increased (Table 23). The results of GC/MS analysis of the radioactive peaks extracted from the chromatogram shown in Figure 35 are presented in Table 24. The GC/MS data substantiate that the compound in the bile ($R_f = 0.46$) of PCA-exposed trout was PCP.

The studies with PCP and PCA indicate that they are taken up rapidly from water by rainbow trout and assimilated into various tissues. Although the amounts of ^{14}C in the tissues of trout exposed to ^{14}C-PCP and PCA cannot be taken as true steady-state tissue concentrations, these short-term exposures indicate that the concentration of PCA in fat was much higher than that of PCP. The data in the elimination studies agree with this finding, and the high retention time of ^{14}C from PCA (half-life in days) probably reflects the high lipid solubility of PCA as compared with PCP. On the other hand the concentration of ^{14}C derived from PCP is much higher in the bile--both in the uptake studies and the elimination studies--than the ^{14}C derived from PCA. We have reported previously that phenols, such as Bayer 73 and TFM, can be conjugated and exposed in bile in high concentrations in rainbow trout (Statham and Lech 1975). This latter observation probably accounts for the high concentration of ^{14}C derived from PCP that is found in rainbow trout bile in this study.

The identification of conjugated PCP in bile of rainbow trout exposed to ^{14}C-PCA indicates that rainbow trout are capable of demethylating PCA *in vivo*. This finding is supported further by the capability of PBO--an inhibitor of mixed-function oxidases--to decrease the amount of PCP glucuronide in bile while concomitantly increasing the amount of unchanged PCA; the finding is also in agreement with studies which have confirmed the dealkylation of several foreign compounds in fish *in vivo* (Hansen et al. 1972, Olson et al. 1977). Although the difference in magnitude of biliary excretion of ^{14}C derived from PCA as opposed to PCP is difficult to explain, several reasons are plausible. PCP is a phenol, which can be conjugated directly with glucuronic acid and excreted in bile, while PCA must first be demethylated before it is conjugated with glucuronic acid, and demethylation may be the overall rate-limiting step in biliary excretion. The high concentration in tissues of ^{14}C from PCA as opposed to that of PCP also must be considered. The transfer of PCA from its tissue depots to sites of metabolism and excretion (gills, liver, kidney) may well be the overall limiting factor in elimination of PCA.

1,2,4-Trichlorobenzene--

Small rainbow trout were exposed to ^{14}C-1,2,4-trichlorobenzene (TCB) in a static system or in a continuous flow delivery system in a similar manner to the exposures previously described for ^{14}C-naphthalene and 2-methylnaphthalene. The results of the short-term static exposure are presented in Table 25, while those for long-term exposure are presented in

TABLE 23. EFFECT OF PBO ON DISTRIBUTION OF ^{14}C IN BILE OF RAINBOW TROUT EXPOSED TO ^{14}C-PCA

Conditions[a]	Number	Bile volume, ml	^{14}C concentration as PCA, µg/ml	Total excreted as PCA, ng	Metabolite distribution					
					PCP-glucuronide		PCP		PCA	
					ng	%	ng	%	ng	%
Control	8	0.082 ± 0.02	3.78 ± 0.57	310	257	83	6	2	47	15
PBO (1 mg/liter)	7	0.098 ± 0.01	2.06 ± 0.21[b]	202	85	42	10	5	107	53

[a] Control trout and trout which had been exposed to 1 mg/liter of PBO for 24 h were placed in separate tanks containing 0.05 mg/liter of ^{14}C-PCA. After 12 h the fish were sacrificed.

[b] Significantly different from control, $p < 0.01$.

TABLE 24. GC/MS ANALYSIS OF CHROMATOGRAPHICALLY-SEPARATED ^{14}C FROM β-GLUCURONIDASE-TREATED BILE FROM RAINBOW TROUT EXPOSED TO ^{14}C-PCA[a]

Sample	Molecular ion (M+) observed	(M+) - CH_3
PCP standard	264, 266, 268, 270	
PCA standard	278, 280, 282, 284	263, 265, 267, 269
R_f = 0.46, untreated	264, 266, 268, 270	
R_f = 0.46, methylated	278, 280, 282, 284	263, 265, 267, 269
R_f = 0.85, untreated	278, 280, 282, 284	
R_f = 0.85, methylated	278, 280, 282, 284	263, 265, 267, 269

[a] See Glickman et al. (1977) for experimental details.

Table 26. In both experiments bile contained by far the highest levels of ^{14}C of any of the tissues examined. The highest biliary concentrations of ^{14}C observed in the short-term study were about 100 to 250 times the initial water concentration of ^{14}C, while in the long-term study biliary ^{14}C was maintained at 500 to 1,400 times the average water concentration of ^{14}C for the duration of the exposure, and the ratio was about 100 for the entire elimination period.

TABLE 25. UPTAKE AND ELIMINATION OF ^{14}C-1,2,4-TCB
BY RAINBOW TROUT: SHORT-TERM EXPOSURE[a]

	Time, h	^{14}C-TCB compound and metabolites, µg/g or ml of tissue			
		Muscle	Liver	Blood	Bile
Exposure	2	0.60[b] ±0.03	1.40 ±0.45	0.23 ±0.06	0.7 ±0.2(4)
	4	0.78 ±0.05	1.13 ±0.09	0.37 ±0.09	2.2 ±0.3(3)
	8	0.91 ±0.07	1.83 ±0.21	0.59 ±0.08	1.9 ±0.3(3)
Elimination	10	0.61 ±0.09	1.36 ±0.16	0.08 ±0.06	4.3 ±1.6
	32	0.13 ±0.02	0.35 ±0.05	0.00	0.9 ±0.3
	56	0.04 ±0.02	0.07 ±0.03	0.00	0.6 ±0.1(4)

[a] Static exposure, initial concentration of TCB 0.018 mg/liter.

[b] Average ± S.E. of values from five fish which were sacrificed at each time. In some cases the bile could not be sampled; the number of samples of bile is indicated in parentheses.

Levels of ^{14}C in muscle, liver, and blood dropped rapidly during the elimination period in both studies, but in the long term study the ^{14}C remaining in the muscle and liver disappeared more slowly. The half-lives of elimination are presented in Table 27. Because of the abrupt change in rate of loss of tissue ^{14}C in the long term study after 1 day of elimination, 1 day of elimination was selected as the basis of comparison of elimination rates in the two studies. The rates of elimination of ^{14}C were similar for muscle and liver during the first 24 h of elimination. Although this same rate of elimination was maintained for the second 24 h period after short term exposure, the rate of elimination of ^{14}C after long term

TABLE 26. UPTAKE AND ELIMINATION OF ^{14}C-1,2,4-TCB BY RAINBOW TROUT: LONG-TERM EXPOSURE[a]

	Time, day	^{14}C-TCB and metabolites, μg/g or ml of tissue			
		Muscle	Liver	Blood	Bile
Exposure	1	0.82[b] ±0.07	3.12 ±0.11	0.37 ±0.09	10.9 ±2.5
	2	1.27 ±0.15	3.76 ±0.19	0.56 ±0.14	14.4 ±1.1
	4	1.44 ±0.21	4.19 ±0.16	0.53 ±0.11	
	7	1.51 ±0.17	4.21 ±0.36	0.86 ±0.26	13.0 ±2.1
	11	2.80 ±0.32	8.47 ±0.36	1.78 ±0.35	16.4 ±2.8(4)
	18	1.60 ±0.15	4.92 ±0.26	1.51 ±0.23	9.1 ±5.8(3)
	35	1.54 ±0.20	7.01 ±0.52	0.34 ±0.14	24.2 ±4.1
Elimination	36	0.18 ±0.02	0.58 ±0.09	0.00	5.0 ±4.5(3)
	37	0.16 ±0.02	0.53 ±0.07	0.00	1.5 ±0.8(3)
	39	0.13 ±0.01	0.40 ±0.08	0.00	2.4(1)
	42	0.13 ±0.01	0.36 ±0.07	0.00	1.6 ±0.4
	57	0.11 ±0.02	0.28 ±0.07	0.00	1.0 ±0.4
	71	0.05 ±0.01	0.25 ±0.03	0.00	1.6 ±0.3

[a] Exposure performed using a continuous flow delivery system; average water concentration of TCB is 0.018 mg/liter.

[b] Average ± S.E. of values from five fish which were sacrificed at each time, except day 71 when seven fish were sacrificed. In some instances the bile volumes were inadequate for sampling; the number of samples of bile is indicated in parentheses.

exposure dropped to < 1% of the initial rate.

Blood ^{14}C dropped to undetectable levels in both studies by 24 h of elimination. The data after 2 h of elimination in the short term study indicated a half-life of < 1 h for blood ^{14}C.

Information on metabolites of ^{14}C-1,2,4-TCB in bile and tissue extracts is presented in Section 4 (Effect of Inducers on Disposition of Organic Chemicals in Rainbow Trout).

TABLE 27. ELIMINATION OF TRICHLOROBENZENE BY RAINBOW TROUT

Exposure	Elimination	Half-life of elimination Muscle	Liver
8 h	0-24 h	9.0 h	10.5 h
	0-48 h	10.9 h	10.5 h
35 days	0-24 h	7.7 h	6.7 h
	1-36 days	36.1 days	32.2 days

Di-2-ethylhexylphthalate (DEPH)

Phthalate esters are a class of chemicals widely used and commonly present in water and aquatic species. These chemicals can be made more polar by hydroysis, but at the same time they may be more extensively metabolized. The di-2-ethylhexyl ester is the most common and was selected for study *in vivo* and *in vitro*.

Metabolism and Disposition *In Vivo* --

Three groups of rainbow trout were exposed to 0.5 ppm of ^{14}C-DEHP as described under Methods (Melancon and Lech 1976). The first group of 14 70-100 g fish yielded 191 µg of DEHP equivalents in the pooled bile. Subsequent groups of 34 70-100 g fish and 48 180-240 g fish yielded 769 and 1634 µg of DEHP equivalents in the pooled bile from each exposure, respectively. The tissue distribution of ^{14}C expressed as DEHP equivalents for the second exposure is given in Table 28. One-half of the total DEHP and metabolites in the fish was found in the bile, and the concentration was over 200 times that of DEHP originally present in the water.

Metabolites were isolated from pooled bile as shown in Figure 36. Bile from each exposure was pooled and desalted with an XAD-2 column. Most of the data presented here pertains to bile from the third exposure. The XAD-2 eluate contained 90.2% of the ^{14}C applied to the column. An aliquot of the

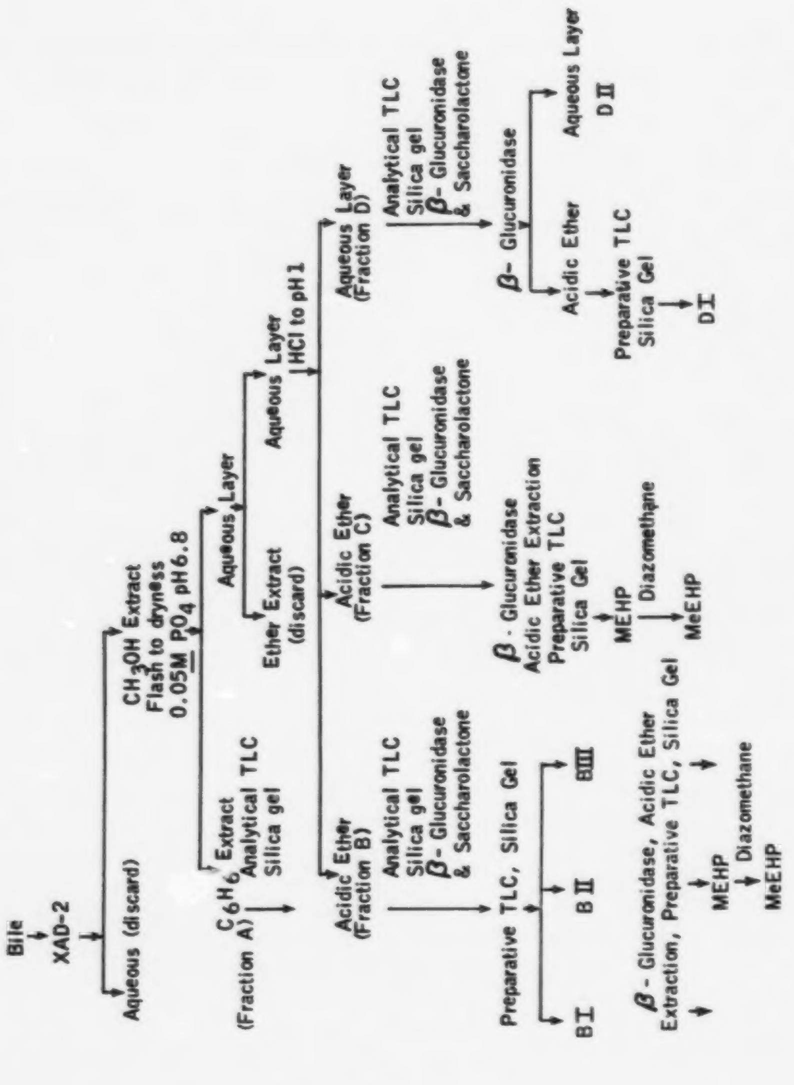
Bile
XAD-2
Aqueous (discard)
CH3OH Extract
Flash to dryness
0.05M PO4 pH6.8
C6H6 Extract
(Fraction A)
Analytical TLC
Silica gel
Aqueous Layer
Ether Extract
(discard)
Aqueous Layer
HCl to pH1
Acidic Ether
(Fraction B)
Analytical TLC
Silica gel
β- Glucuronidase
& Saccharolactone
Preparative TLC, Silica Gel
BI
BII
BIII
MEHP
Diazomethane
MeEHP
β - Glucuronidase, Acidic Ether
Extraction, Preparative TLC, Silica Gel
Acidic Ether
(Fraction C)
Analytical TLC
Silica gel
β - Glucuronidase
& Saccharolactone
β - Glucuronidase
Acidic Ether Extraction
Preparative TLC
Silica Gel
MEHP
Diazomethane
MeEHP
Aqueous Layer
(Fraction D)
Analytical TLC
Silica gel
β- Glucuronidase
& Saccharolactone
β- Glucuronidase
Acidic Ether
Aqueous Layer
Preparative TLC
Silica Gel
DI
DII

Figure 36. Flow diagram for the isolation and purification of biliary DEHP and metabolites.

XAD-2 eluate was chromatographed with chloroform:methanol:acetic acid (143:7:2)(solvent 2). As shown in the uppermost graph of Figure 37, this material was much more polar than the DEHP starting material.

TABLE 28. TISSUE LEVELS OF DEHP (AND/OR METABOLITES) FOLLOWING 24 H EXPOSURE TO AQUEOUS DEHP AT AN INITIAL LEVEL OF 0.5 PPM[a]

Tissue	Tissue weight, g	Total DEHP, μg	DEHP, μg/g
Bile	0.2	22.6	111.4
Blood	0.4	0.2	0.5
Liver	0.7	2.1	3.0
Gills	2.4	5.4	2.3
Fins	1.3	1.7	1.3
Viscera	4.9	2.6	0.5
Kidney	0.4	0.2	0.5
Skin	6.2	1.2	0.2
Head	6.4	2.5	0.4
Carcass	49.5	5.5	0.1
Whole fish	72.3	44.0	0.6

[a] Values for bile, blood, and liver are based on the pooled samples from 34 fish. Values for all other tissues are based on the pooled samples from two fish.

Fractionation was performed as described in Figure 36 and resulted in radioactive fractions A, B, C, and D which contained 1.5, 62.5, 18, and 18%, respectively, of the XAD-2 eluate radioactivity. Suitably-sized aliquots of Fractions A through D were chromatographed with solvent system 2. As shown in Figure 35, Fraction A contained mostly unchanged DEHP (metabolite AI) and a small amount of mono-2-ethylhexyl phthalate (MEHP) (metabolite AII), both at such low levels that they were not observed in the earlier TLC of the XAD-2 eluate. Fractions B, C, and D consisted of more polar materials.

It was anticipated that DEHP metabolites might be present in bile, and the solubility and TLC mobility of the bile fractions suggested the presence of conjugates. To check for the presence of glucuronides, aliquots of Fractions B, C, and D were incubated with β-glucuronidase with and without saccharo-1,4-lactone. As the data in Table 29 show, the high percentage of the radioactivity in each fraction that remained near the origin was reduced by 25-90% by β-glucuronidase hydrolysis. In all fractions this hydrolysis was reduced substantially by the presence of saccharo-1,4-lactone. Each of the fractions was examined in more detail.

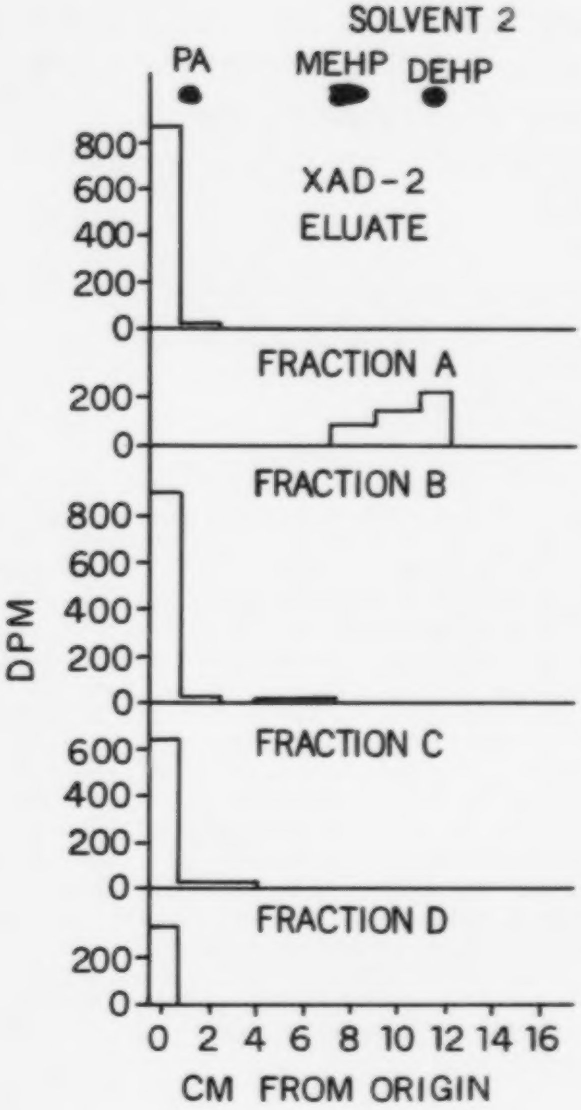

Figure 37. Thin-layer chromatography of fractionated rainbow trout bile. Solvent 2 (chloroform:methanol:acetic acid of 143:7:2). The mobilities of phthalic acid (PA), MEHP, and DEHP are indicated by the dark spots.

TABLE 29. β-GLUCURONIDASE HYDROLYSIS OF MAJOR BILE METABOLITE FRACTIONS[a]

Fraction	Total dpm remaining at origin, %		
	Control	+ β-glucuronidase	+ β-glucuronidase + saccharo-1,4-lactone
B	97.4	8.2	62.6
C	92.5	60.6	87.6
D	96.3	72.2	95.0

[a] Total incubation volume was 0.5 ml containing 100 units of β-glucuronidaseand 0.001 *M* saccharo-1,4-lactone where indicated. Solvent system 2 was used for TLC.

Preparative TLC of Fraction B with chloroform:methanol:acetic acid (5:1:1) (solvent 4) resulted in three radioactive peaks. The major peak (metabolite BII) had an R_f value of 0.61, and the minor peaks (metabolites BI and BIII) had R_f values of 0.44 and 0.85, respectively. Samples of each of the three metabolites were incubated with β-glucuronidase. These three metabolites were chromatographed with solvent 4 before and after incubation (Figure 38). Metabolites BI and BII were both changed to less polar compounds by β-glucuronidase action, but metabolite BIII was unaffected. In this solvent system metabolite BIII and hydrolyzed metabolites BI and BII all co-chromatographed with MEHP. Because metabolites BI and BII chromatographed differently from each other as glucuronides, we expected that two different compounds would be released by β-glucuronidase hydrolysis. This was confirmed by chromatography with solvent 2. As shown in Figure 39, hydrolyzed metabolite BII co-chromatographed with MEHP, whereas both metabolites BIII and hydrolyzed BI were more polar. Hydrolyzed metabolite BII, after reaction with diazomethane, co-chromatographed with MeEHP, as shown in Figure 40. Metabolite BII accounted for about 56% of bile radioactivity and appeared to be MEHP glucuronide. Metabolites BIII and hydrolyzed BI were pooled because of their similar mobilities in TLC and are referred to later.

Fraction C was characterized by use of the same techniques described for the study of fraction B. This fraction was composed mainly of MEHP glucuronide (metabolite CI).

Fraction D was incubated with β-glucuronidase, followed by acidification and ether extraction. Although this fraction--after acidification--had been extracted exhaustively with ether during the original fractionation, this procedure now yielded a compound (hydrolyzed Metabolite DI) which was similar to Metabolites BIII and hydrolyzed BI. Figure 41 shows that the R_f value of hydrolyzed Metabolite DI is different from that of non-hydrolyzed Fraction D and similar to that of MEHP in solvent 4, whereas its mobility in solvent 2 is definitely different from

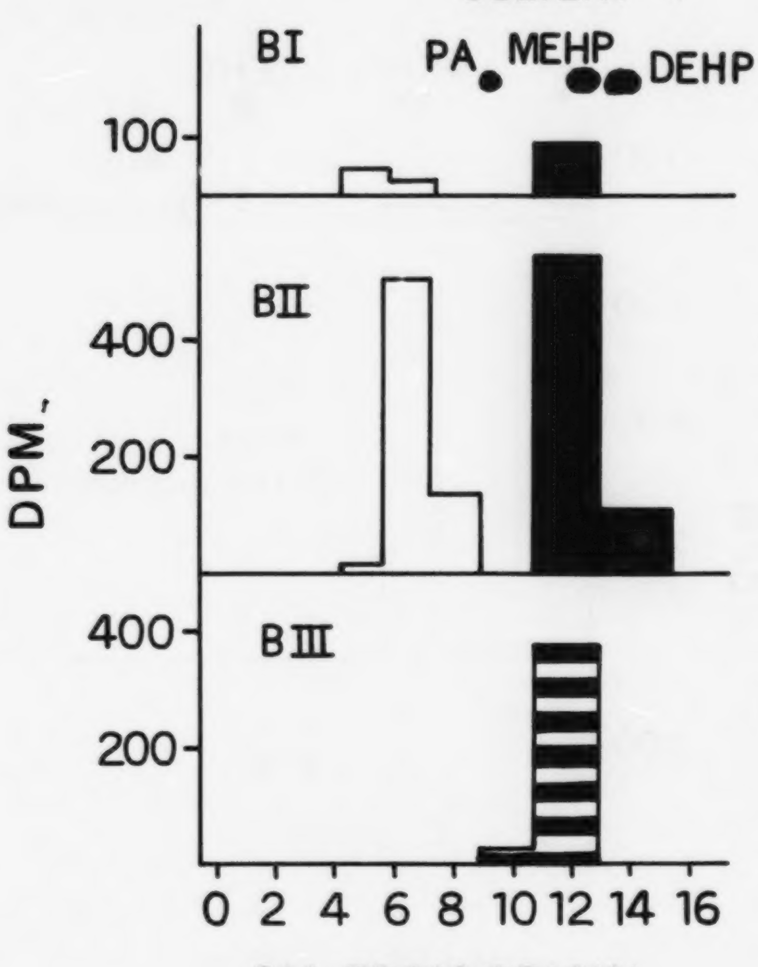

Figure 38. Effect of β-glucuronidase hydrolysis on the TLC mobility of Metabolites BI, BII and BIII. Mobilities of Metabolites BI and BII before and after β-glucuronidase hydrolysis are represented by open and closed bars, respectively. The striped bar for Metabolite BIII indicates that the R_f value of this metabolite was unchanged by incubation with β-glucuronidase. Solvent 4 is chloroform:methanol:acetic acid (5:1:1). The mobilities of PA, MEHP, and DEHP are indicated by dark spots.

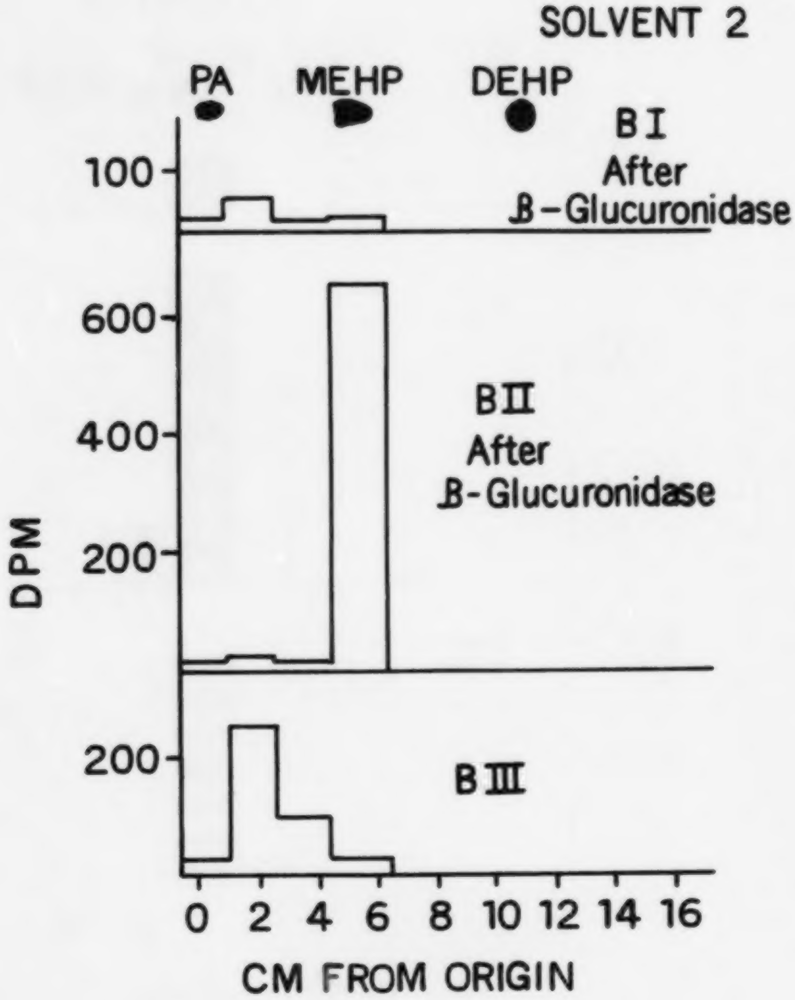

Figure 39. Thin-layer chromatography of Metabolites BI, BII and BIII after incubation with β-glucuronidase. Solvent 2 is chloroform: methanol:acetic acid (143:7:2). The mobilities of PA, MEHP, and DEHP are indicated by dark spots.

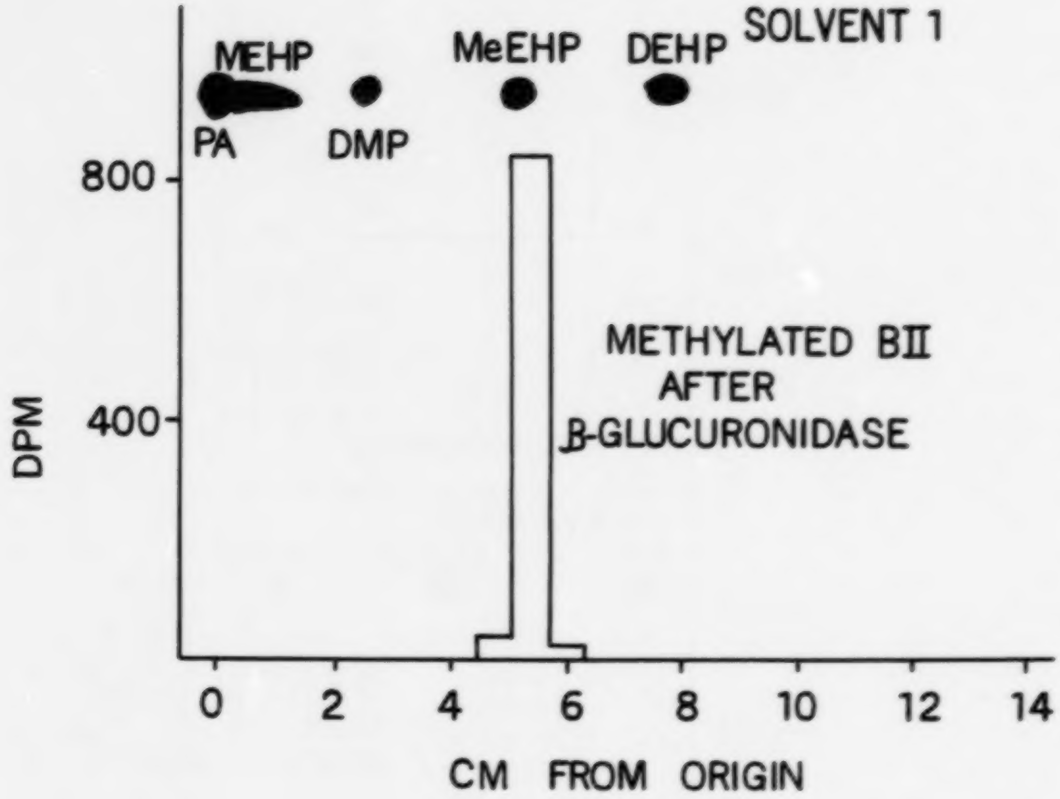

Figure 40. Thin-layer chromatography of Metabolite BII, methylated following β-glucuronidase hydrolysis. Solvent 1 (benzene:ethyl acetate (19:1). The mobilities of PA, MEHP, DMP, MeEHP, and DEHP are indicated by dark spots.

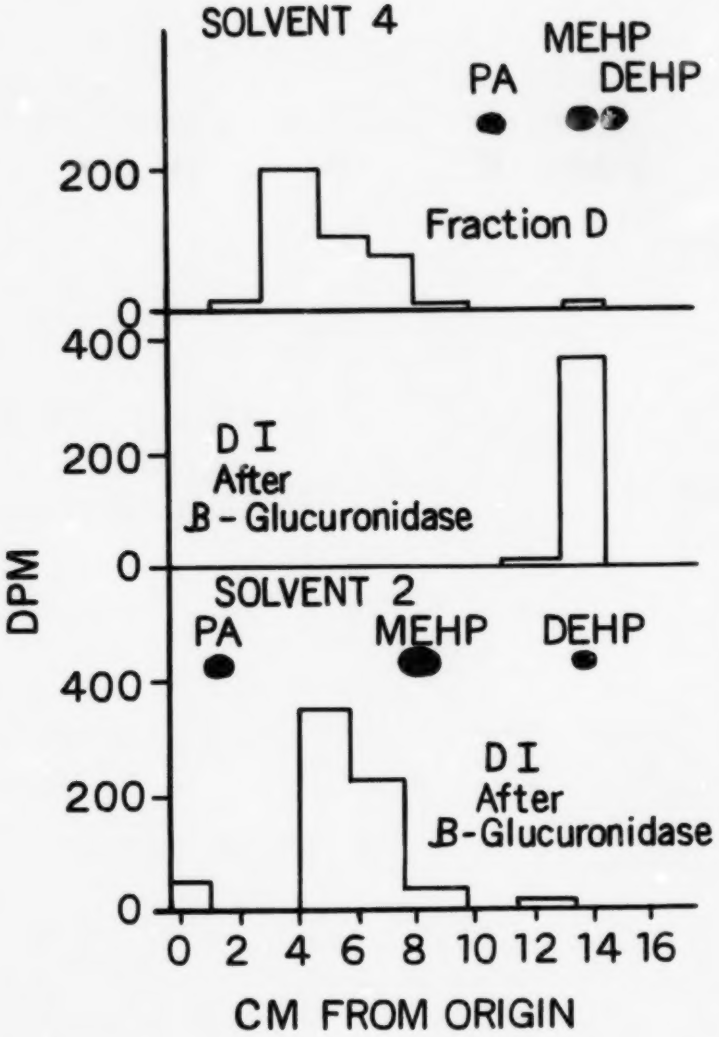

Figure 41. Thin-layer chromatography of Fraction D (top graph), and of the acidic ether extract of this fraction after β-glucuronidase hydrolysis (hydrolyzed Metabolites DI), lower graphs, in two solvents. Solvent 4 is chloroform:methanol:acetic acid (5:1:1) and was utilized to obtain upper graphs. Solvent 2 is chloroform:methanol:acetic acid (143:7:2) and was utilized to obtain the lower graph. The mobilities of PA, MEHP and DEHP are indicated by dark spots.

that of MEHP and similar to that of metabolite BIII. A small amount of PA (DIII) also was found.

Because of their similar mobilities in TLC, metabolite BIII and hydrolyzed metabolites BI and DI were combined. Although reaction with diazomethane decreased the polarity of this material, it was still at least as polar as MEHP.

The radioactive material that remained in the aqueous phase after β-glucuronidase treatment of Fraction D and extraction contained 9% of the total bile radioactivity. This material (metabolite DII) has been characterized only by its relatively high polarity in TLC, resistance to β-glucuronidase hydrolysis, and lack of extraction from aqueous solution by the organic solvents used in this study.

A summary of the approximate amounts of DEHP and metabolites found in the various bile fractions is presented in Table 30. Unchanged DEHP accounted for only 1% of the bile radioactivity. About 83.5% of the radioactivity was present as glucuronide conjugates--namely, MEHP glucuronide, (72%); PA glucuronide, (2%); and polar metabolite glucuronide, (9.5%). In addition, about 9% of bile radioactivity was present as very polar material.

TABLE 30. DISTRIBUTION OF ^{14}C IN FRACTIONATED TROUT BILE[a]

Compound	Designation	Fraction, %			
		A	B	C	D
DEHP	AI	1			
MEHP	AII	0.5			
MEHP-glucuronide	BII,CI	56	16		
PA-glucuronide	DIII				2
Polar metabolite	BIII	3			
Polar metabolite-glucuronide	BI,DI	2.5			7
Polar-nonextractable material	DII				9

[a] Fractions as described in text. ^{14}C percentages are based on radioactivity in XAD-2 eluate.

In a similar experiment, fractions of radioactivity from the XAD-2 eluates of bile from the first two exposure groups extractable into benzene or acidic ether before and after β-glucuronidase hydrolysis were pooled. Preparative TLC with several solvent systems yielded six radioactive peaks. One of these co-chromatographed with DEHP, several co-chromatographed with MEHP, and another was more polar than MEHP and was similar to the combined polar metabolites BIII and hydrolyzed BI and DI described earlier. These metabolites were subjected to GC/MS and the results are shown in Table 31. The GC peaks were located with the radioactive monitor to insure that actual metabolites were being identified. In agreement with the TLC data, the GC/MS results obtained with sample 1 were consistent with the presence of unchanged DEHP. The results with methylated samples 2, 3, 4, and 5 were consistent with the presence of MeEHP, indicating MEHP as the major metabolite. Sample 6, after methylation, chromatographed as more polar than any of the three standard diesters and lacked a peak at m/e 112, corresponding to the 2-ethylhexyl moiety, suggesting a metabolite with a modified sidechain.

TABLE 31. THE GC/MS ANALYSIS OF PHTHALATE METABOLISM

	GC-RAM elution temperature, °C		MS (m/e+)	
	5% DEGS	3% OV-7	Base peak	Secondary peak
Standards				
DEHP	225	231	149	167
MeEHP	203	189	163	149
DMP	174	133	163	
Samples				
1	225		149	167
2[a]	203	189	163	149
3[a]	203			
4[a]	203	189		
5[a]	203	190	163	149
6[a]	240	240	163	149

[a] Methylated.

These studies show that rainbow trout readily can convert DEHP to MEHP. Table 32 compares the results of this study of DEHP and metabolites in trout bile and of the study of whole catfish extracts following 24-h exposure reported by Stalling et al. (1973). The 83.5% of biliary phthalates found as conjugates (see Table 30) is > 14% of conjugated phthalates present in whole fish extracts reported by Stalling et al. (1973).

TABLE 32. DEHP AND METABOLITES IN RAINBOW TROUT AND CATFISH

Compound	Rainbow trout,[a] % in bile	Catfish,[b] % in whole fish
DEHP	1	14
MEHP	0.5	66
MEHP-glucuronide	72	13.7
PA	0	4
PA-glucuronide	2	0.3
Other	21.5	2

[a] Rainbow trout data summarized from Table 30.
[b] Catfish data from Stalling et al. (1973).

Although it was expected that the level of conjugates in bile would be higher than in whole fish, such a large difference was not expected. Because bile radioactivity represents about 50% of total fish radioactivity in this study, the finding of 83.5% biliary conjugates is equivalent to > 40% conjugates on a whole-fish basis, even if no conjugates are present in the remainder of the fish. Expressed in terms of quantities of each phthalate metabolite, the results agree more closely. Total MEHP accounted for about 72.5% in rainbow trout bile as compared to 80% in whole catfish. The large difference in total conjugates between the two species suggests either that the two species differed in rate of conjugation or that enterohepatic circulation and redistribution occurred in the catfish studies.

Total PA was low in both species. Significant differences were observed, however, in the amounts of unchanged DEHP and of very polar metabolites. It was expected that bile would contain mostly DEHP metabolites, whereas the other tissues would be more likely to contain unmodified DEHP. However, the 21.5% of very polar metabolites found in trout bile is equivalent to > 10% on a while-fish basis, i.e., five times the value reported for catfish. The difference in amounts of these very polar metabolites might be caused by species differences but also could occur because the trout were exposed to 0.5 ppm DEHP whereas the catfish were exposed to only 0.001 ppm. The effect of exposure level on metabolite patterns is under investigation.

Possible pathways for the formation of DEHP metabolites found in rainbow trout bile are illustrated in Figure 42. From the large amount of MEHP glucuronide present in rainbow trout bile, the sequence DEHP→MEHP→MEHP glucuronide was obviously the major metabolic pathway. The PA glucuronide could have arisen by either of the indicated pathways. The unidentified polar glucuronide could have been formed in a variety of ways. The mass spectral data suggest that the ring structure was intact whereas the 2-

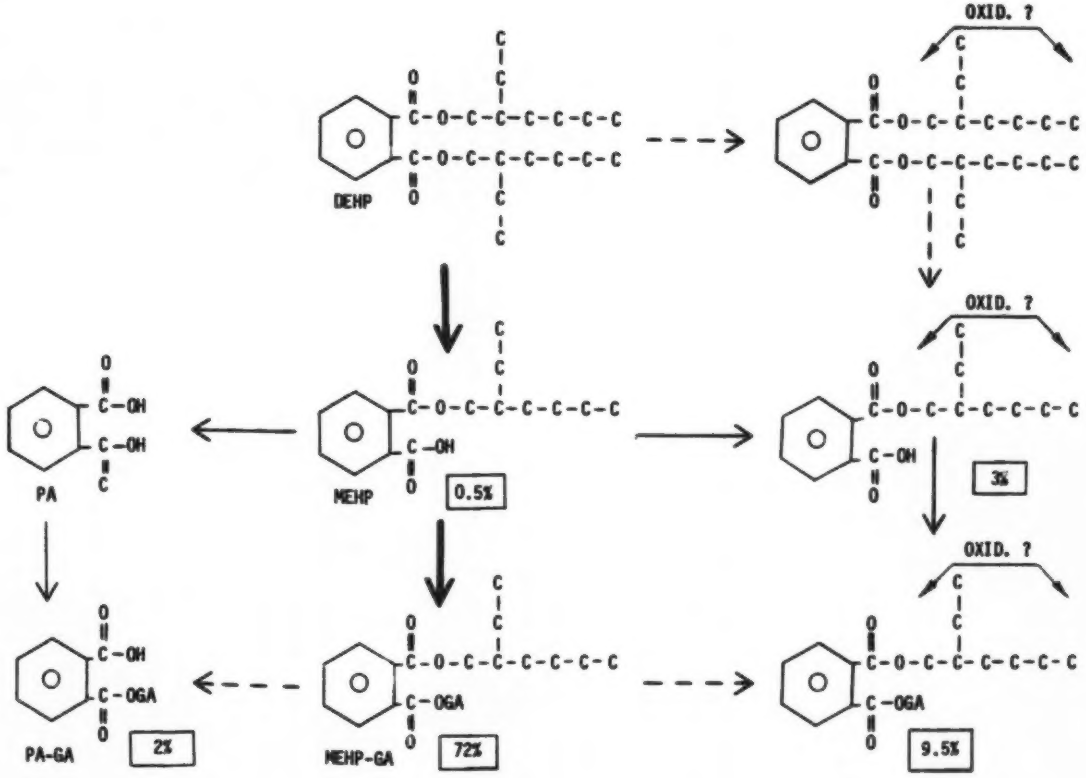
OXID. ?
DEHP
MEHP
0.5%
3%
PA
PA-GA
2%
MEHP-GA
72%
9.5%
C-OH
C-OGA

Figure 42. Pathways for DEHP metabolism by rainbow trout. The percentages in boxes refer to radioactivity in XAD-2 eluate. Heavy arrows indicate major pathway, light arrows indicate likely pathways, and dashed arrows indicate possible pathways.

ethylhexyl moeity was modified. Several of the studies of DEHP metabolism in rats have indicated that DEHP is hydrolyzed to MEHP before the 2-ethylhexyl moeity is modified. Because glucuronide formation generally is considered to facilitate excretion, MEHP glucuronide is probably not further metabolized by oxidation. These two considerations suggest that the pathway for the formation of this metabolite is DEHP→MEHP→oxidized MEHP→oxidized MEHP glucuronide.

Metabolism of DEHP in Rainbow Trout *In Vitro*--

DEHP metabolism was initially studied *in vitro* by utilizing the 2000 *g* supernatant fraction of trout liver homogenates prepared in 0.154 *M* KCl (Melancon and Lech 1977). Representative radioscans of thin-layer chromatograms of extracts of incubations of carboxyl-labeled ^{14}C-DEHP with the 2000 *g* supernatant fraction are shown in Figure 43. The lower tracing shows that when NADPH was not added to the incubation mixture, MEHP was the predominant metabolite formed. Addition of NADPH, shown in the upper tracing, resulted in the appearance of two additional radioactive peaks. The peak adjacent to that of MEHP was designated polar metabolite 1 and the peak near the origin, polar metabolite 2, inasmuch as they have not been identified.

A summary of the amounts of MEHP and polar metabolite 1 formed in several such experiments is presented in Table 33. Without added NADPH, metabolism consisted almost entirely of hydrolysis of DEHP to MEHP. When NADPH was added the production of polar metabolite 1 increased substantially. The increase in polar metabolites, a decrease in MEHP accumulation, and an increase in total DEHP metabolism resulting from the addition of NADPH are shown in Table 33, and are also apparent in Table 34 and Figure 44 and Figure 45. Piperonyl butoxide blocked hydrolysis and oxidation of DEHP. Hydrolysis or oxidation of DEHP was not observed in incubations containing heat-inactivated liver fractions.

TABLE 33. METABOLISM OF DEHP BY TROUT LIVER HOMOGENATES[a]

Additions	MEHP formed nmoles/h/g of liver	Polar metabolite I formed nmoles/h/g of liver
0	2.84 ± 0.68	0.27 ± 0.14
+ NADPH	1.62 ± 0.41	2.43 ± 0.68
+ PBO	0.14 ± 0.00	0.00 ± 0.00
+ NADPH, PBO	0.00	0.00

[a] Incubations were performed as described in text. Each incubation contained 0.034 moles ^{14}C-DEHP in a total volume of 1 ml. Values presented are the mean ± SEM for three separate homogenates.

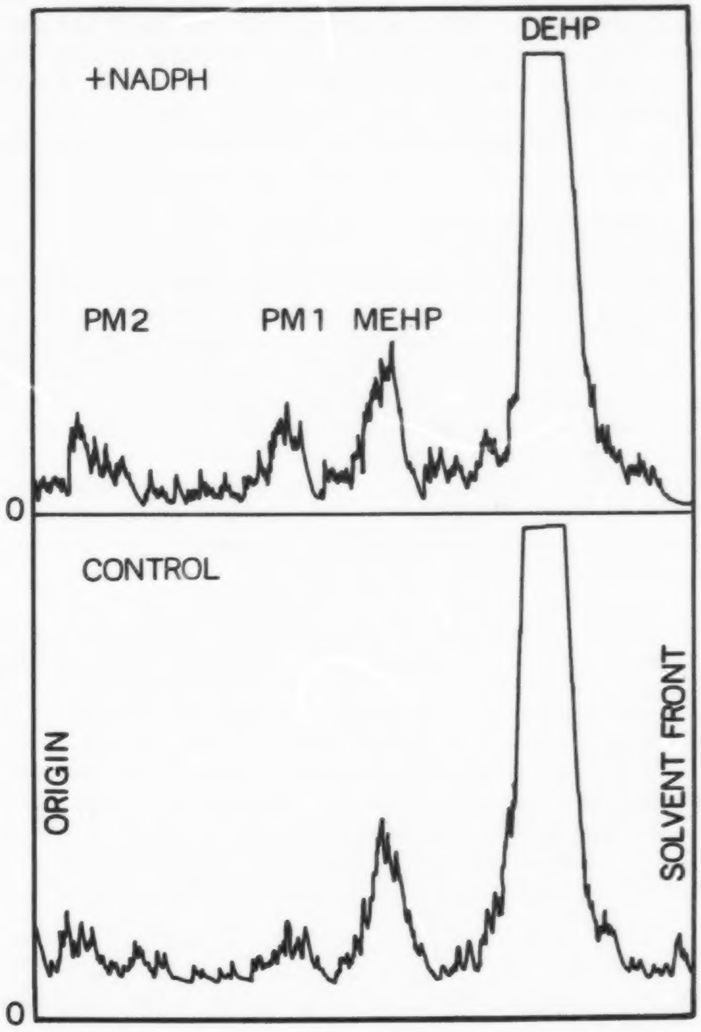

Figure 43. Metabolite patterns following incubation of ^{14}C-DEHP with trout liver homogenate. The 2000 *g* supernatant fraction of trout liver homogenate was incubated as described in the text with 0.010 µmol of ^{14}C-DEHP in a total volume of 2 ml for 1 h with or without added NADPH. The ether extracts from the acidified incubation media were subjected to TLC with chloroform:methanol:acetic acid (143:7:2). DEHP and MEHP were located with chromatographed standards. PM1 and PM2 represent Polar Metabolites 1 and 2.

TABLE 34. METABOLISM OF DEHP BY SUBCELLULAR FRACTIONS OF TROUT LIVER HOMOGENATES AND BY TROUT BLOOD SERUM[a]

		Nanomoles of metabolite formed/h/g of liver by addition of				
Experiment	Fraction	NADPH	PBO	MEHP	Polar metabolite 1	Polar metabolite 2
1	2,000 *g* supernatant	0	0	3.91	0.53	0.20
		X	0	3.11	3.38	1.66
1	Mitochondria	0	0	1.99	0.07	0.07
		X	0	2.19	0.73	0.33
		X	X	0.13	0.13	0.07
1	Microsomes	0	0	4.51	0.20	0.13
		X	0	2.23	2.93	1.41
		X	X	0.13	0.26	0.07
1	100,000 *g* supernatant	0	0	4.13	0.07	0.07
2	2,000 *g* supernatant	0	0	3.84	0.20	0.07
		0	X	0.26	0.00	0.00
2	Mitochondria	0	0	2.52	0.26	0.07
		0	X	0.20	0.00	0.00
2	Microsomes	0	0	2.50	0.00	0.07
		0	X	0.26	0.00	0.07
2	100,000 *g* supernatant	0	0	3.15	0.07	0.07
		0	X	0.33	0.07	0.07
	Nanomoles of metabolite formed/h/0.8 ml of serum					
	Serum	0	0	0.61±0.11	0.01±0.00	0.04±0.03
		0	X	0.03±0.01	0.00	0.00

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[a] Incubations of 1-h were performed as described under Methodology. Each incubation contained 0.010 μmoles ^{14}C-DEHP in a total volume of 2 ml. Serum samples from each of three fish were incubated separately. Values for serum are the mean ± SEM.

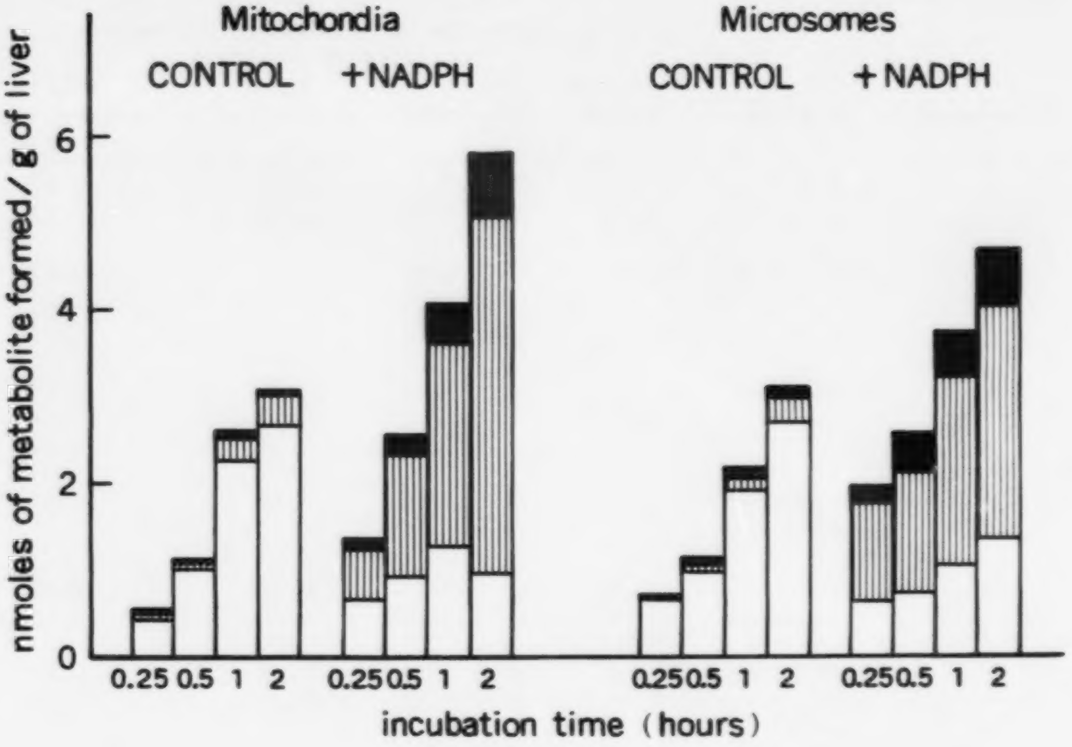

Figure 44. Influence of time on metabolism of ^{14}C-DEHP by trout liver mitochondrial and microsomal fractions. Incubations, as described in the text, contained 0.10 μmol of ^{14}C-DEHP in a total volume of 2 ml. Mitochondria equivalent to 0.254 g of liver or microsomes equivalent to 0.361 g of liver were used in each incubation. Open bars represent MEHP, striped bars Polar Metabolite 1, and solid bars Polar Metabolite 2. Each column represents an individual incubation.

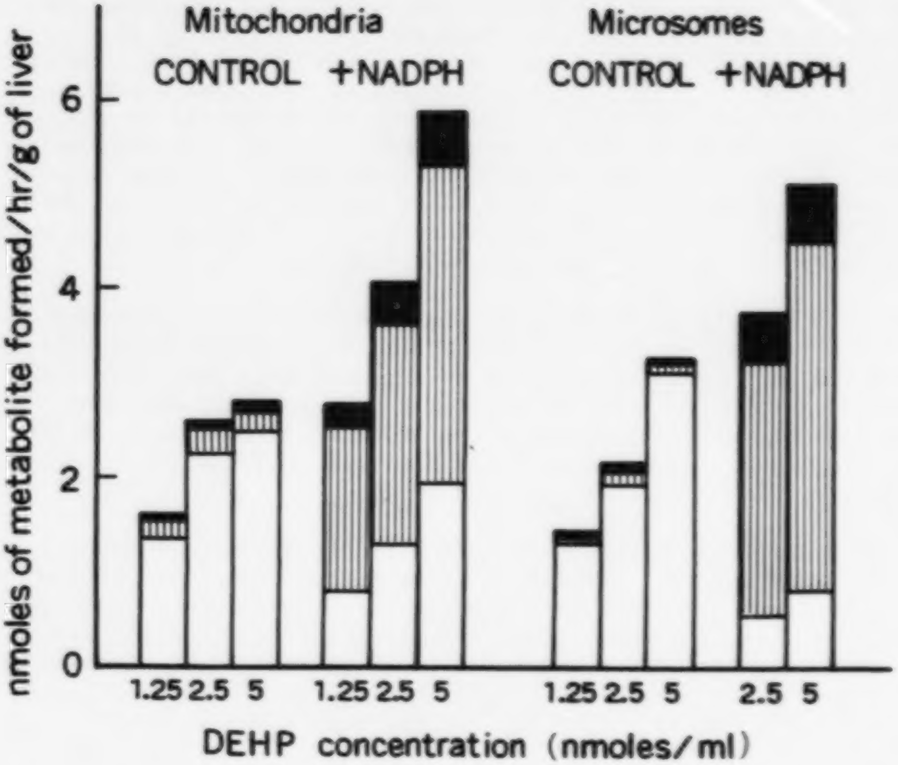

Figure 45. Influence of DEHP concentration on metabolism of ^{14}C-DEHP by trout liver mitochondrial and microsomal fractions. Incubations as described in the text, were done in a total volume of 2 ml for 1 h. Mitochondria equivalent to 0.254 g of liver of microsomes equivalent to 0.361 g of liver were used in each incubation. Open bars represent MEHP, striped bars Polar Metabolite 1 and solid bars Polar Metabolite 2. Each column represents an individual incubation.

In subsequent experiments the 2000 *g* supernatant fraction was further fractionated as described in the section on methodology to determine which organelles were reponsible for DEHP hydrolysis and oxidation. Because blood--including fish blood--is known to contain esterase activity (Augustinsson 1959), the ability of trout serum to catalyze DEHP hydrolysis was also investigated. Ring-labeled ^{14}C-DEHP was used in these and all following experiments. The results of two experiments comparing the metabolism of DEHP by the 2000 *g* supernatant fraction and by the "mitochondrial", "microsomal", and 100,000 *g* supernatant fractions are presented in Table 34. All the liver homogenate fractions showed significant hydrolysis of DEHP to MEHP, which was inhibited by PBO. Incubation of the mitochondrial or microsomal fraction with DEHP and NADPH gave results similar to those obtained with the 2000 *g* supernatant fraction. Although not shown in Table 34, added NADPH had little effect on DEHP metabolism by the 100,000 *g* supernatant fraction. Serum contained DEHP-hydrolytic activity that also was blocked by PBO.

Figure 44 and Figure 45 show the time-course and substrate dependence of DEHP metabolism by the "mitochondrial" and "microsomal" fractions with and without added NADPH. In addition to demonstrating the increased production of metabolites with increasing incubation time and DEHP concentration, the data show clearly the effects of addition of NADPH, i.e., a greatly increased production of polar metabolites 1 and 2 with reduced accumulation of MEHP.

The ability of PBO to block formation of the oxidized metabolites of DEHP was not surprising, but its inhibition of the hydrolysis of DEHP to MEHP--both in liver fractions and in serum--was unexpected. To investigate this effect further, the metabolism (hydrolysis) of a different ester, 2,4-DBE, by these liver fractions and blood serum was measured. Studies have shown that 2,4-DBE is hydrolyzed readily by fish *in vivo* (Rodgers and Stalling 1972, Statham and Lech 1976). The data in Table 35 show that trout liver subcellular fractions readily hydrolyze 2,4-DBE and that PBO substantially inhibits the hydrolysis. Also, PBO inhibited the serum-catalyzed hydrolysis of 2,4-DBE.

In a number of experiments in which the metabolism of DEHP by the 2000 *g* supernatant fraction and by subfractions prepared from it was measured, the hydrolysis of DEHP by the 2000 *g* supernatant fraction was not great enough to account for the sum of DEHP esterase activities present in the mitochondrial, microsomal, and 100,000 *g* supernatant fractions.

In order to investigate this observation, a 2000 *g* supernatant fraction of liver homogenate was centrifuged at 100,000 *g*, and this pellet was resuspended in a volume of 0.154 *M* KCl equal to 25% of the volume of the supernatant fluid removed. Varying amounts of this suspension and of the 100,000 *g* supernatant fraction were incubated in the usual manner, i.e., separately and in combination with each other. The results presented in Table 36 show no linear increase in amount of DEHP hydrolyzed with increasing amounts of the particulate suspension and show that the hydrolytic activity of the combined fractions does not equal the sum of the hydrolytic activities of the fractions individually.

TABLE 35. METABOLISM OF 2,4-DBE BY SUBCELLUAR FRACTIONS OF TROUT LIVER HOMOGENATES AND BY TROUT BLOOD SERUM[a]

			Nanomoles of 2,4-DBE hydrolyzed/h/g of liver by addition of			
Experiment	NADPH	PBO	2,000 *g* supernatant	Mitrochondria	Microsomes	100,000 *g* supernatant
1	0	0	101.4	92.6	107.4	83.8
	X	0	100.0	79.1	100.0	
	X	X	28.4	29.7	25.0	
2	0	0	97.3	103.4	86.5	108.1
	0	X	33.1	19.6	28.4	30.4
			Nanomoles of 2,4-DBE hydrolyzed/h/0.08 ml of serum			
Serum	0	0		16.0 ± 0.4		
	0	X		2.2 ± 0.3		

[a] The 1-h incubations were performed as described under Methodology. Each incubation contained 0.02 µmoles of ^{14}C-2,4-DBE in a total volume of 2 ml. Serum samples from each of three fish were incubated separately. Values for serum are the mean ± SEM.

Because of the occurrence of DEHP-metabolizing activity in the various liver homogenate fractions and because of the close similarity between mitochondrial and microsomal metabolism of DEHP shown in Figure 42 and Figure 43, these fractions needed to be characterized further. For this reason, the activity of marker enzymes and DEHP metabolizing enzymes and protein concentration were determined for the 2000 *g*, 10,000 *g*, 100,000 *g* pellets and 100,000 *g* supernatant fraction of trout liver homogenates. The enzyme activities measured were succinic dehydrogenase, glucose 6-phosphatase, DEHP esterase (MEHP formation without added NADPH), and DEHP oxidase (formation of metabolites more polar than MEHP with added NADPH). The relative specific activity for each enzyme activity measured in each fraction versus the percentage of total protein present in each fraction as described by deDuve et al. (1955) is presented in Figure 46.

The data concerning the distribution of the mitochondrial marker (succinic dehydrogenase) and the microsomal marker (glucose 6-phosphatase) indicate that the standard rat liver fractionation technique does not produce a satisfactory separation of organelles when trout liver is used. Based on succinic dehydrogenase activity, most mitochondria were present in the 2000 *g* pellet, with a smaller fraction in the so-called "mitochondrial fraction." Some microsomes (glucose 6-phosphatase) were distributed in the 2000 *g* pellet, but high relative specific activity was observed in the "mitochondrial" as well as in the "microsomal" fractions. In addition, DEHP

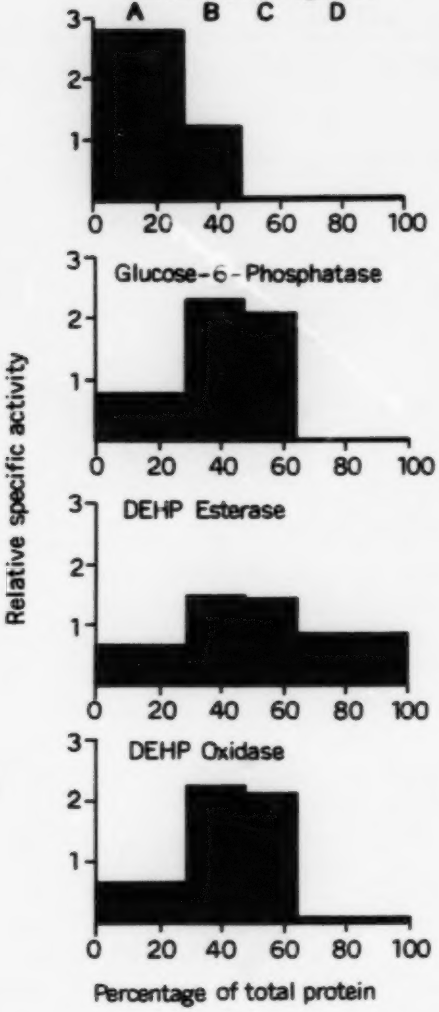

Figure 46. Distribution of marker enzymes and DEHP-metabolizing enzymes in trout liver homogenate fractions. DEHP esterase and DEHP oxidase were each measured by 1 h incubations of 0.010 µmol of ^{14}C-DEHP in a total volume of 2 ml. A. 2000 *g* pellet: B. 10,000 *g* pellet: C. 100,000 *g* pellet: D. 100,000 *g* supernatant fraction. Relative specific activity - percentage of total activity:percentage of total protein.

TABLE 36. HYDROLYSIS OF DEHP BY RECOMBINED TROUT LIVER HOMOGENATE FRACTIONS[a]

Liver equivalent of 100,000 *g* supernatant fraction, mg	Hydrolysis rate (nmole/h) for liver equivalent of mitochondria and microsomal fractions of, 0	33 mg	66 mg	132 mg
0	0.03	0.39	0.46	0.59
68	0.45	0.70 (0.89)	0.94 (0.91)	0.74 (1.04)
136	1.11	0.75 (1.50)	0.94 (1.57)	0.92 (1.70)

[a] Incubation conditions were as described in the text except that a highly concentrated resuspension of the mitochondrial plus microsomal pellet was used so that the volumes of this suspension plus 100,000 *g* supernatant fraction could be kept to 0.8 ml. Each reaction vessel contained 0.01 μmole of ^{14}C-DEHP in a volume of 2 ml. Incubation was for 1 h at 22°C. All incubations containing 136 mg (liver equivalent) of 100,000 *g* supernatant fraction were performed in duplicate, wherease the other data are individual values. Expected values are given in parentheses.

oxidase activity is high in the fractions that contain most of the glucose 6-phosphatase and low in the 2000 *g* pellet, which contains most of the mitochondria. The DEHP esterase activity was high in the fractions which were high in glucose 6-phosphatase and DEHP oxidase activities, and substantial esterase activity occurred in the 100,000 *g* supernatant fraction, which contained little if any mitochondrial or microsomal markers.

The results show that rainbow trout liver homogenates have the ability to metabolize DEHP. Without added NADPH, metabolism consisted mainly of hydrolysis of DEHP to MEHP. Whereas with added NADPH metabolism shifted from production of MEHP to more polar metabolites and more total DEHP was metabolized.

The production of polar metabolites in the presence of added NADPH could depend on oxidation of DEHP itself or on oxidation of MEHP. The reduced level of MEHP after incubations with NADPH as compared to those without NADPH suggests that the polar metabolites appear at the expense of MEHP. In most incubations < 20% of the DEHP was metabolized; thus, sufficient DEHP was present as substrate for the hydrolytic reaction. These data suggest that oxidation of MEHP occurs subsequent to hydrolysis of DEHP. Studies of the *in vivo* metabolism of DEHP in rats demonstrated that except for a small amount of phthalic acid, the metabolies were either MEHP or oxidized derivatives of MEHP (Albro et al. 1973, Daniel and Bratt 1974). Similar results were observed with other phthalate esters (Albro and Moore 1974, Williams and Blanchfield 1975). The absence of oxidized diester metabolites suggests that metabolism of these phthalate esters consists of hydrolysis to a monoester followed by oxidation of the remaining side chain, or that any oxidized diester produced is very rapidly hydrolyzed to the oxidized monoester. The results of *in vitro* metabolism of ^{14}C-DEHP by channel catfish liver microsomes reported by Stalling et al. (1973) indicate that added ^{14}C-MEHP was not further metabolized. Identification of the polar metabolites of DEHP formed by liver fractions from rainbow trout and catfish may explain the apparent differences between these studies.

Both DEHP and 2,4-DBE were hydrolyzed by all of the liver homogenate fractions and by serum; hydrolysis of both compounds was inhibited by PBO. Other researchers have reported that PBO can inhibit the microsome-catalyzed hydrolysis of the pesticides parathion and diazinon but that this hydrolysis required NADPH and oxygen (Nakatsugawa and Dahm 1967, Nakatsugawa et al. 1969). In this study, NADPH was not required for DEHP hydrolysis, but hydrolysis by the liver fractions and by serum was inhibited by PBO. Although no apparent explanation exists for this inhibition, a similar effect has been observed *in vivo* with dioctyl phthalate in *Culex* and *Estigmene acrea* larvae (Sanborn et al. 1975).

When rates of hydrolysis of DEHP and 2,4-DBE by liver homogenates and by serum were compared per gram of liver and per milliliter of serum, the rate of hydrolysis by serum was twice that of liver. Most of the experiments in the current study were performed at approximately 2 ppm DEHP. *In vivo* exposure of trout to DEHP at 0.5 ppm in water for 24 h gave 3.0 ppm in liver and 0.5 ppm in blood (Melancon and Lech 1976). Thus, the DEHP levels *in vivo* and in these *in vitro* studies of DEHP metabolism were similar.

When increasing amounts of the particulate fractions were added to the 100,000 *g* supernatant fraction, a linear increase in DEHP hydrolysis was not observed. At the higher level of 100,000 *g* supernatant fraction used, addition of the particulate fraction actually caused an inhibition of hydrolysis. Because of the lipophilic nature of DEHP, DEHP may be nonspecifically bound by these organelles. In fact, the *in vivo* accumulation of DEHP by beef heart mitochondria has been reported (Nazir et al. 1971).

Although DEHP esterase activity occurred in all the liver subcellular fractions, the distribution of the microsomal marker--glucose 6-phosphatase--obviously demonstrated that DEHP esterase was distributed with microsomes and was also present in the 100,000 *g* supernatant fraction. Although DEHP oxidase was also present in several fractions, this activity was highest in fractions that were highest in glucose 6-phsophatase activity. The studies of Carter et al. (1974) with rat liver subcellular fractions demonstrated the hydrolysis of DEHP in "mitochondrial" and "microsomal" fractions and a trace of activity in the cytosol. Because the mitochondrial fraction in this latter report was sedimented by an 18,000 *g* centrifugation for 20 min, microsomal contamination probably occurred in this fraction, and the DEHP metabolizing activity attributed to mitochondria may well reside in microsomes. The accumulation of DEHP *in vivo* in beef heart mitochondria (Nazir et al. 1971) also would suggest that mitochondria do not metabolize DEHP.

The distribution of DEHP esterase activity in rainbow trout liver subcellular fractions is interesting because it differs from that found in rat liver preparations. Several reports (Underhay et al. 1956, Shibko and Tappel 1964, Schwark and Ecobichon 1968, Ljungquist and Augstinsson 1971) give the total esterase activity in cytosol as from < 1 to almost 20%. The higher values generally are reported when microsomal contamination of the cytosol is present (Underhay et al. 1956, Schwark and Ecobichon 1968). In the experiment shown in Figure 44, 34.6% of total DEHP esterase activity and 0.4% of total glucose 6-phosphatase activity were found in the 100,000 *g* supernatant fraction, and in a subsequent experiment the values were 30.7% and 1.6%, respectively. Thus, residual microsomal activity in the 100,000 *g* supernatant fraction cannot account for the high esterase activity found in this fraction from trout liver. Possible explanations of this activity are 1. that two different esterases can hydrolyze DEHP and 2. a single esterase may be partially solubilized from microsomes during preparation of liver homogenates.

Effect of PBO on Metabolism of Di-2-ethylhexylphthalate *In Vivo* and *In Vitro*--

The inhibiting effect of PBO on the metabolism of DEHP and 2,4-DBE *in vitro* was not explained by the reported effects of PBO. Additional experiments were performed to examine the *in vitro* effects in more detail and to determine whether this PBO inhibition of DEHP metabolism by fish also occurred *in vivo* (Melancon et al. 1978).

Radiochromatogram tracings of the ether extracts of incubations of ^{14}C-DEHP with the 2000 *g* supernatant of trout liver homogenates with or without PBO are shown in Figure 47. The upper tracing shows the major metabolite peaks--MEHP and polar metabolite 1--which were present when the incubations contained added NADPH. The actual percentages of total ^{14}C on the plate were MEHP (6.2%); polar metabolite 1 (13.3%); and polar metabolite 2 (1.5%). The lower tracing does not show any metabolite peaks from the incubation with PBO present; the DEHP peak represented 99.5% of the total radioactivity found on the plate.

A summary of the effects of 2×10^{-3} *M* PBO on DEHP and 2,4-DBE metabolism in incubations with trout liver fractions and with serum is presented in Table 37. The inhibition of hydrolysis of these compounds by PBO in all fractions is readily apparent as is the inhibition of NADPH-dependent DEHP oxidation by PBO in liver fractions.

The effect of 9×10^{-3} *M* PBO on DEHP metabolism by trout liver homogenate and serum is shown in Table 38. Even though the concentration of PBO was lowered by a factor of over 22, hydrolysis of DEHP by serum and

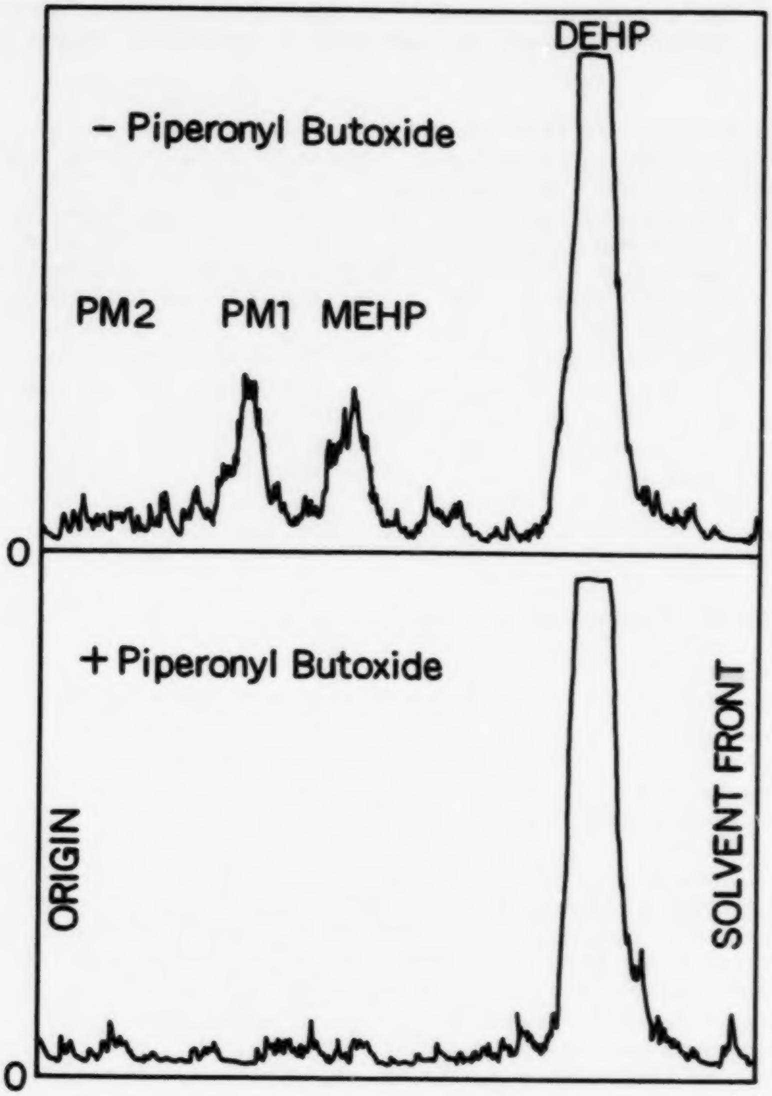

Figure 47. Effect of PBO on the metabolism of DEHP by trout liver homogenates. The 2000 *g* supernatant fraction of a rainbow trout liver homogenate was incubated in phosphate buffer with 5 μ*M* ^{14}C-DEHP and 2 m*M* NADPH. ^{14}C extracted following the incubation was examined by TLC on silica-gel coated plates using $CHCl_3$:MeOH:HOAc (143:7:2) as solvent system. Tracings were obtained using a radiochromatogram scanner.

TABLE 37. EFFECT OF PBO ON METABOLISM OF DEHP AND 2,4-DBE BY TROUT LIVER SUBCELLULAR FRACTIONS AND SERUM[a]

Tissue fraction	Additions		Metabolites of DEHP formed, nmole/h/g			2,4-DBE hydrolyzed nmole/h/g
	NADPH (2m*M*)	PBO (2m*M*)	MEHP	Polar metabolite 1	Polar metabolite 2	
Liver homogenate 2,000 *g* supernatant	0	0	6.49 ± 1.53	0.35 ± 0.07	0.15 ± 0.03	99.3
	0	X	0.26 ± 0.04^b	0.03 ± 0.02^b	0.03 ± 0.00^b	33.1
	X	0	3.49 ± 0.38	6.84 ± 2.01	1.43 ± 0.11	100.0
	X	X	0.20 ± 0.01^b	0.02 ± 0.00^b	0.02 ± 0.00^b	28.4
Liver homogenate 100,000 g pellet	0	0	3.93 ± 0.62	0.08 ± 0.05	0.11 ± 0.01	96.9
	0	X	0.20 ± 0.08^b	0.04 ± 0.02	0.03 ± 0.02^b	28.4
	X	0	3.10 ± 0.47	4.07 ± 0.64	1.03 ± 0.20	100.0
	X	X	0.12 ± 0.01^b	0.11 ± 0.08^b	0.04 ± 0.02^b	25.0
			Metabolites of DEHP formed nmole/h/g			2,4-DBE hydrolyzed nmole/h/g
Serum	0	0	0.61 ± 0.11			16.4 ± 0.04
	0	X	0.03 ± 0.01^b			2.2 ± 0.03^b

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[a] Tissues were incubated at 22°C for 1 h with 5 μ*M* DEHP or 10 μ*M* 2,4-DBE. Values shown are mean ± SEM of at least three separate tissue preparations except for hydrolysis of 2,4-DBE by liver fractions, for which only two experiments were performed.

[b] Significantly different from control (zero PBO), $p < 0.05$.

liver homogenate and oxidation of DEHP by liver homogenate were reduced by about 90.

TABLE 38. EFFECT OF PIPERONYL BUTOXIDE ON METABOLISM OF DEHP BY TROUT LIVER HOMOGENATE AND BLOOD SERUM

Tissue fraction	Additions		Metabolites of DEHP formed nmole/h/g		
	NADPH (2mM)	PBO (90 μM)	Polar MEHP	Polar metabolite 1	metabolite 2
Liver homogenate 2,000 *g* supernatant	0	0	7.51 ± 0.82	0.22 ± 0.13	0.14 ± 0.02
	0	X	1.05 ± 0.18[b]	0.29 ± 0.18	0.06 ± 0.01[b]
	X	0	1.14 ± 0.54	11.29 ± 0.93	1.41 ± 0.46
	X	X	1.39 ± 0.37	0.64 ± 0.33[b]	0.08 ± 0.01[b]
Serum	0	0	15.96 ± 0.96		
	0	x	1.08 ± 0.12[b]		

[a] Tissue were incubated at 22°C for 1 h with 5 μm DEHP. Data are presented as mean ± SEM for four separate liver preparations and three serum samples.
[b] Significantly different from control (zero PBO), $p < 0.05$.

Despite this inhibition by PBO of the *in vitro* metabolism of DEHP (oxidation and hydrolysis) and 2,4-DBE (hydrolysis), we could not conclude that PBO would necessarily have the same effects *in vivo*. Consequently, the effect of exposure of trout to PBO upon *in vitro* metabolism of DEHP was investigated. Liver homogenates were prepared from control fish and from fish preexposed to 1 mg/liter PBO for 24 h. The data presented in Table 39 show a reduced production of the metabolites of DEHP by liver homogenates from the fish preexposed to PBO. In the absence of added NADPH the production of MEHP from DEHP was reduced, while in the presence of NADPH the production of polar metabolites was reduced, suggesting that oxidation and hydrolysis of DEHP were inhibited.

Another series of experiments was designed to determine whether exposure of rainbow trout to PBO affected the disposition or metabolism of DEHP *in vivo*. Control fish were exposed to 0.07 mg/liter ^{14}C-DEHP for 24 h, and fish preexposed to 1 mg/liter PBO for 24 h were subsequently coexposed to 0.07 mg/liter ^{14}C-DEHP plus 1 mg/liter PBO for 24 h. The levels of phthalates present in selected tissues expressed as microgram DEHP per gram are presented in Table 40. Fish pretreated with PBO had muscle levels of phthalate twice those of control fish and bile levels 50% those of control fish. The concentration of phthalate also was elevated significantly in blood in the PBO-treated fish. To determine whether the phthalates accumulating in muscle were DEHP or metabolites, pooled muscle samples from control and PBO-exposed fish from each experiment were extracted with acetone and the extracted ^{14}C was examined by TLC. The results presented in Table 41 show that while almost 50% of the total phthalates present in muscle of control fish was found to be MEHP, the value for MEHP in PBO-exposed fish was < 25% of the total phthalate residue.

TABLE 39. METABOLISM OF DEHP BY 2000 X G SUPERNATANT OF LIVER HOMOGENATES FROM CONTROL TROUT AND TROUT PREEXPOSED TO PBO[a]

Treatment	Assay conditions NADPH (2 mM	Metabolites of DEHP formed nmole/h/g MEHP	Polar metabolite 1	Polar metabolite 2
Control	0	9.16 ± 1.58	0.72 ± 0.50	0.11 ± 0.05
	+	3.15 ± 0.63	7.68 ± 0.57	1.57 ± 0.61
Piperonyl	0	3.95 ± 0.21[b]	0.15 ± 0.09	0.06 ± 0.01
butoxide (1 mg/liter)	+	2.35 ± 0.31	2.63 ± 1.28[b]	0.15 ± 0.04

[a] Tissues were incubated at 22°C for 1 h with 5 M DEHP. Each value is the mean ± SEM for three liver homogenates.

[b] Significantly different from control, $p < 0.02$.

TABLE 40. EFFECT OF PBO ON ACCUMUATION OF ^{14}C-DEHP IN VARIOUS TISSUES OF RAINBOW TROUT *IN VITRO*[a]

Treatment	^{14}C concentration, μg/g DEHP Muscle	Blood	Bile	Liver
Control	0.021 ± 0.003	0.142 ± 0.017	51.4 ± 5.5	0.86 ± 0.08
PBO (1 mg/liter)	0.041[b] ± 0.006	0.234[b] ± 0.019	26.2 ± 2.8[b]	1.08 ± 0.15

[a] Control trout and trout which had been exposed to 1 mg/liter of PBO for 24 h were exposed respectively to 0.07 mg/liter ^{14}C-DEHP and to 0.07 mg/liter ^{14}C-DEHP plus 1 mg/liter of PBO for 24 h.

[b] Significantly different from control, $p < 0.01$.

TABLE 41. EFFECT OF PBO ON ACCUMULATION OF DEHP AND MEHP IN MUSCLE OF RAINBOW TROUT *IN VIVO*[a]

Experiment	Treatment	Total ^{14}C, % As DEHP	Total ^{14}C, % As MEHP	Concentration, μg/g As DEHP	Concentration, μg/g As MEHP
1	Control	46.6	41.8	0.014	0.012
	PBO (1 mg/liter)	76.5	18.4	0.042	0.010
2	Control	45.8	37.1	0.006	0.005
	PBO (1 mg/liter)	70.2	26.0	0.020	0.007

[a] Control trout and trout which had been exposed to 1 mg/liter of PBO for 24 h were exposed respectively to 0.07 mg/liter ^{14}C-DEHP and to 0.07 mg/liter ^{14}C-DEHP plus 1 mg/liter of PBO for 24 h. Muscle ^{14}C was characterized by TLC.

When these percentages were applied to the total phthalate concentrations in muscle, MEHP levels were similar in the control and PBO-exposed fish while the differences in DEHP account for the difference in total muscle phthalates.

The ability of PBO to block DEHP oxidation by trout liver *in vitro* corresponds to the known effects of PBO to inhibit microsomal oxidations in mammals (Casida et al. 1966). The effect of PBO *in vitro* to block the hydrolysis of DEHP and 2,4-DBE by trout liver subcellular fractions and trout serum was not anticipated, based on reported actions of PBO. The inhibitory effect of PBO on the microsomal-catalyzed hydrolysis of parathion and related compounds has been reported (Nakatsugawa and Dahm 1967, Kamienski and Murphy 1971), but the proposed pathway of parathion metabolism requires oxidation dependent on NADPH and O_2 prior to hydrolysis.

Preexposure of trout to PBO resulted in reduced oxidation and hydrolysis of DEHP by the 2000 *g* supernatant of trout liver homogenates, indicating that these effects of PBO might be occurring *in vivo*

Preexposure of trout ot PBO *in vivo* modified the disposition and matabolism of DEHP *in vivo*, and the major effects observed were decreased biliary phthalates and increased muscle phthalates in PBO-preexposed trout as compared to controls. Examination of the phthalates in muscle revealed that the level of DEHP in this tissue from PBO-preexposed fish was three times that of controls.

Thus, the *in vivi* and *in vitro* effects of PBO on DEHP metabolism by rainbow trout appear to be similar. As discussed previously, a decreased oxidation of DEHP is consistent with the known effects of PBO to inhibit microsomal oxidations. The reported inhibition of DEHP hydrolysis differs from the indirect effect of PBO on hydrolysis of parathion in that the hydrolysis of DEHP appears not to require NADPH and in that non-microsomal hydrolysis of DEHP by the 100,000 *g* supernatant of liver homogenates and by serum also was inhibited. Piperonyl butoxide has been shown to inhibit or stimulate microsomal enzymes depending on the duration of treatment (Kamienski and Murphy 1971). Because of the immediacy of esterase inhibition seen in the *in vitro* experiments in this report, this effect is obviously not related to long-term processes involving enzyme synthesis. The two types of experiments reported herein involving *in vivo* exposures of trout to PBO yielded data which were consistent with the *in vitro* results. Liver homogenates prepared from PBO-exposed trout had lower DEHP esterase activity based upon *in vitro* assays. In addition, the metabolic disposition of DEHP in intact rainbow trout was altered by PBO in a manner which would be consistent with the inhibition of DEHP hydrolysis *in vivo*.

The ability of PBO--which is not considered a direct inhibitor of hydrolysis--to inhibit the hydrolysis of DEHP and 2,4-DBE raises the question of the basis of this inhibition. The structures of DEHP, 2,4-DBE, and PBO (Figure 48) indicate that the three compounds have a benzene ring with at least one long chain substituent containing six or more carbon atoms and oxygens in ester or ether linkage. Similarity also existed among the phthalate moiety of DEHP, the methylenedioxyphenyl moiety of PBO, and the

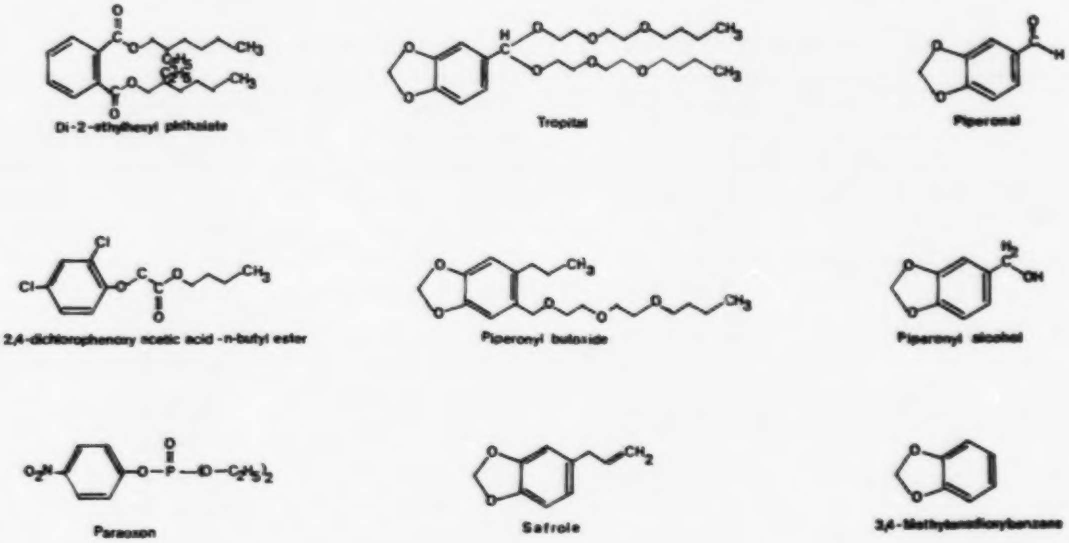
Di-2-ethylhexyl phthalate
Tropital
Piperonal
2,4-dichlorophenoxy acetic acid-n-butyl ester
Piperonyl butoxide
Piperonyl alcohol
Paraoxon
Safrole
3,4-Methylenedioxybenzene

Figure 48. Chemical structures of di-2-ethylhexylphthalate, 2,4,-dichlorophenoxyacetic acid-n-butyl ester, paraoxon and methylenedioxyphenyl compounds.

dichlorophenyl moiety of 2,4-DBE. In order to assess whether the methylenedioxyphenyl group or the side chain of PBO was responsible for this inhibition, several other methylenedioxyphenyl compounds were examined for their abilities to inhibit DEHP hydrolysis *in vitro* (Melancon and Lech 1979). The potent esterase inhibitor paraoxon also was used for comparison.

The hydrolysis of DEHP by serum and by a post-mitochondrial liver fraction was inhibited substantially by paraoxon, PBO, and tropitol (Table 42). The remaining methylenedioxyphenyl compounds had little or no inhibitory effect.

TABLE 42. EFFECT OF MICROSOMAL INHIBITORS ON DEHP HYDROLYSIS

Inhibitor		Serum,[a] nmoles DEHP metabolized	Liver homogenate (8,000 *g* supernate), nmoles DEHP metabolized
Control		1.76 ± 0.16^b	1.26 ± 0.14
Piperonyl butoxide	5×10^{-5} *M*	0.62 ± 0.09^c	0.33 ± 0.10^c
	1×10^{-3} *M*	0.09 ± 0.01^c	0.13 ± 0.01^c
Saffrole	5×10^{-5} *M*	1.72 ± 0.12	1.24 ± 0.06
	1×10^{-3} *M*	1.59 ± 0.22	1.21 ± 0.28
Tropitol	5×10^{-5} *M*	0.68 ± 0.22^c	0.43 ± 0.06^c
	1×10^{-3} *M*	0.06 ± 0.00^c	0.16 ± 0.02^c
Piperonyl alcohol	5×10^{-5} *M*	2.23 ± 0.20	1.40 ± 0.17
	1×10^{-3} *M*	2.52 ± 0.15	1.82 ± 0.13^c
1,3-benzodioxole	5×10^{-5} *M*	1.93 ± 0.10	1.32 ± 0.08
	1×10^{-3} *M*	1.74 ± 0.10	1.16 ± 0.14
Piperonal	5×10^{-5} *M*	1.93 ± 0.16	0.94 ± 0.07
	1×10^{-3} *M*	1.19 ± 0.17^c	1.39 ± 0.11
Paroxon	5×10^{-5} *M*	0.05 ± 0.01^c	0.13 ± 0.00^c
	1×10^{-3} *M*	0.06 ± 0.01^c	0.09 ± 0.01^c

[a] One ml of either a 1-5 dilution of trout serum in 0.010 *M* sodium phosphate (pH 7.2) or the 8,000 *g* supernatant fraction of a 1-4 homogenate of liver in 0.25 *M* sucrose diluted to the original homogenate volume were added to 1 ml of 0.01 *M* potassium phosphate (pH 7.2) containing 10 nmoles of ^{14}C-DEHP and the indicated inhibitor and incubated at 22°C for 1 h.

[b] Data presented are the mean ± S.E. of observation from two experiments, each of which contained three control incubations and two for each inhibitor concentration.

[c] Significantly different from control, $p < 0.025$; those not so labeled, $p > 0.05$.

Additional experiments were performed on inhibition of the metabolism of DEHP by microsomes and by cytosol. The number of inhibitors was reduced by eliminating several examples of methylenedioxyphenyl compounds with short chain substituents. The data (Table 43) show substantial inhibition of DEHP

TABLE 43. EFFECT OF MICROSOMAL INHIBITORS ON DEHP METABOLISM

Inhibitor	Microsomes[a] (-NADPH), nmoles DEHP metabolized	Microsomes (+NADPH)				Cytosol
		nmoles MEHP formed	nmoles PM1 formed	nmoles PM2 formed	nmoles DEHP metabolized	nmoles DEHP metabolized
Control	0.79 ± 0.14[b]	0.44 ± 0.08	1.26 ± 0.17	0.20 ± 0.06	1.90 ± 0.27	0.46 ± 0.05
Piperonyl butoxide	0.29 ± 0.02[c]	0.22 ± 0.03[c]	0.15 ± 0.02[c]	0.03 ± 0.01[c]	0.41 ± 0.06[c]	0.14 ± 0.04[c]
1,3-benzodioxole	0.76 ± 0.11	0.45 ± 0.04	1.04 ± 0.16	0.14 ± 0.06	1.63 ± 0.26	0.40 ± 0.08
Paraoxon	0.05 ± 0.00[c]	0.33 ± 0.04	0.37 ± 0.05[c]	0.29 ± 0.10	1.00 ± 0.17[c]	0.09 ± 0.02[c]
Tropitol	0.28 ± 0.04[c]	0.15 ± 0.01[c]	0.14 ± 0.05[c]	0.04 ± 0.01[c]	0.34 ± 0.08[c]	0.17 ± 0.04[c]

[a] One ml of microsomal suspension or cytosol was added to 1 ml of 0.01 M potassium phosphate (pH 7.2) containing 5×10^{-16} *M* ^{14}C-DEHP, 10^{-4} *M* inhibitor (where indicated) and 2×10^{-3} *M* NADPH (where indicated) were incubated at 22° for 1 h.

[b] Data presented are the mean ± S.E. of observations from two experiments, each of which contained three control incubations and two for each inhibitor.

[c] Significantly different from control, $p < 0.05$.

PM1 and PM2 are polar metabolites 1 and 2.

metabolism by every inhibitor in this set of experiments except 1,3-benzodioxole.

The results suggest that a fortuitous similarity in the structures of DEHP, 2,4-DBE, and PBO is responsible for the inhibition of DEHP hydrolysis by PBO.

EFFECT OF INDUCERS ON DISPOSITION OF ORGANIC CHEMICALS IN RAINBOW TROUT

The preceding study showed that PBO inhibited DEHP metabolism *in vivo* and *in vitro*. Numerous experiments described earlier in this report also demonstrated that a number of chemicals induced hepatic cytochrome(s) P-450 and related enzyme activities in trout. The next step was to determine whether the induction of these enzyme activities affected the metabolism and disposition of xenobiotics by fish.

In our first experiment of this type, control trout and trout injected with 2,3-benzanthracene were exposed to aqueous ^{14}C-methylnaphthalene for 6 h (Statham et al. 1978).

Increased rates of metabolism and elimination of ^{14}C-2-methylnaphthalene *in vivo* were observed after pretreatment of the trout with 2,3-benzanthracene. Figure 49 demonstrates that the rate of appearance of radioactive material in bile was increased dramatically by pretreatment with 2,3-benzanthracene. Interestingly, the initial levels of ^{14}C were higher in livers of induced trout and appeared to be retained longer during the washout period. Pretreatment with 2,3-benzanthracene did not appear to affect significantly the rate of disappearance of radioactive material from blood or muscle.

The TLC of extracts of bile indicated a greater proportion of polar radioactive materials related to 2-methylnaphthalene and less parent compound in bile after induction of monooxygenation by 2,3-benzanthracene (Figure 50). Although the polar metabolites have not yet been characterized, several different compounds appear to occur in the polar materials.

Subsequent experiments to investigate the effects of induction on the disposition and metabolism of foreign chemicals by trout utilized a 24-h exposure to the ^{14}C-labeled chemical without an elimination period (Melancon and Lech 1979).

Trout injected with β-naphthoflavone and vehicle-injected trout showed several differences in tissue levels of ^{14}C following exposure to ^{14}C-naphthalene or ^{14}C-2-methylnaphthalene (Table 44). Biliary levels of ^{14}C in β-naphthoflavone- (βNF) treated fish that had been exposed to ^{14}C-naphthalene were 4.6 times those of control fish, while liver ^{14}C was slightly less than that of control fish and muscle and blood levels of ^{14}C were about half those found in control fish. The biliary ^{14}C from control and NF-treated fish consisted almost entirely of polar material while the ^{14}C present as polar material in muscle and liver from βNF-treated trout were 2.4 and 2.8 times the control, respectively. Biliary levels of ^{14}C in NF-treated fish exposed to ^{14}C-2-methylnaphthalene were 8.2 times those of

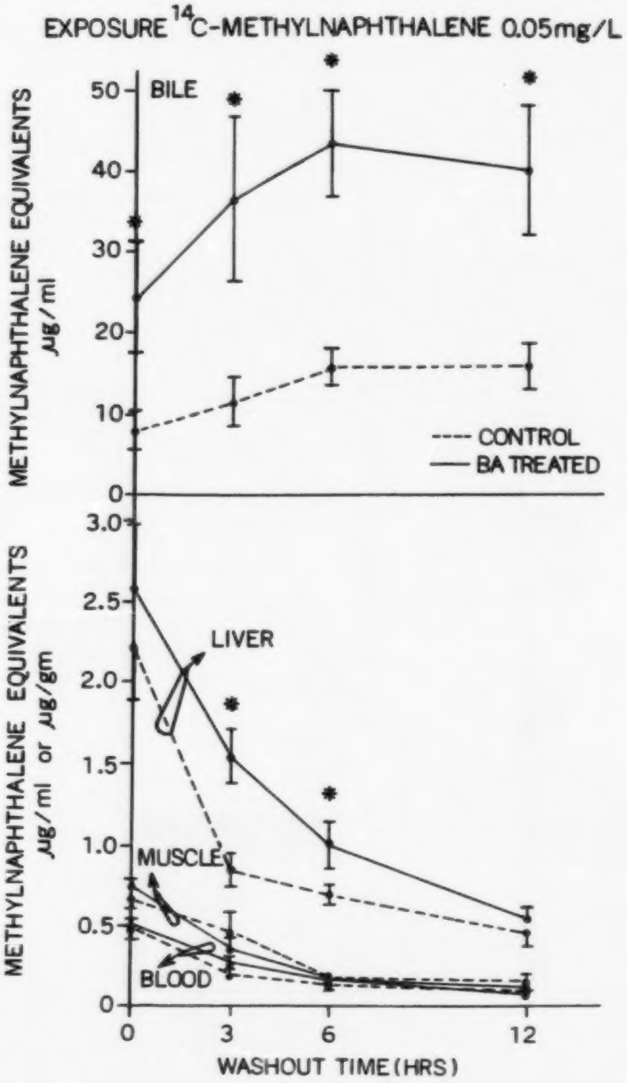

Figure 49. Uptake and elimination of ^{14}C-2-methylnaphthalene derived material in rainbow trout. Each point is the mean ± S.E. (n = 3 in two separate experiments). *$p < 0.05$.

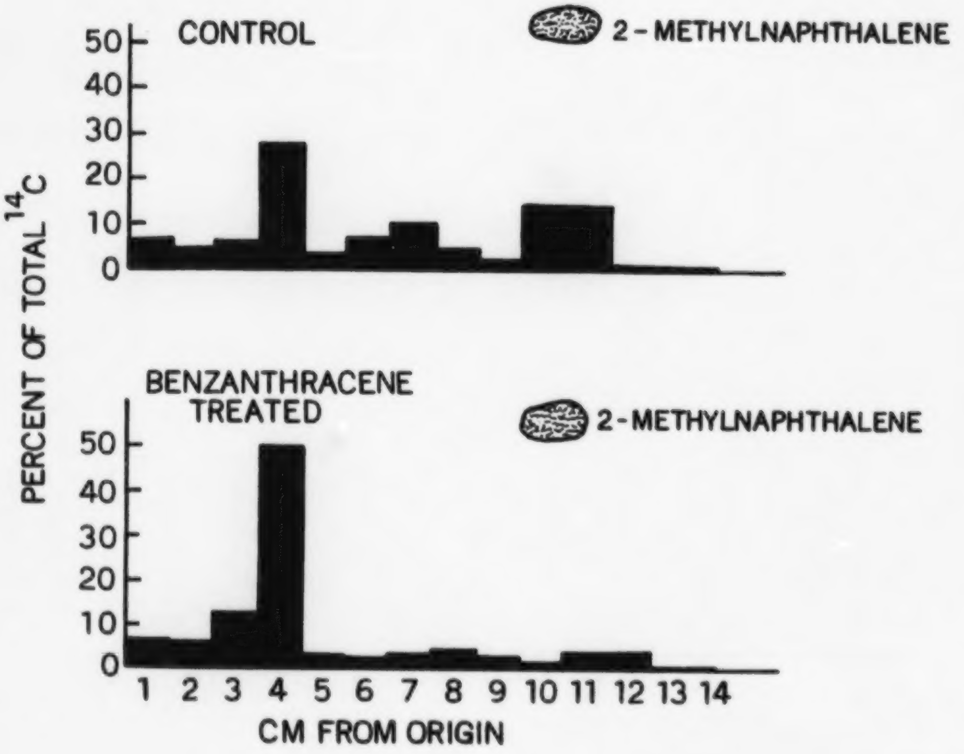

Figure 50. Thin-layer radiochromatographic profile of 2-methylnaphthalene metabolites in trout bile. Solvent: 1-butanol:NH_4OH:water (40:10:50) (organic phase). The shaded spots indicate the mobility of 2-methylnaphthalene.

TABLE 44. EFFECT OF PRE-ADMINISTRATION OF -NAPHTHOFLAVONE ON THE DISPOSITION AND METABOLISM OF ^{14}C-LABELED CHEMICALS IN RAINBOW TROUT

Tissue	Tissue level of parent chemical + metabolites (μg/g or μg/ml)	Metabolites, %	Tissue level of parent chemical + metabolites (μg/g or μg/ml)	Metabolites, %
		Naphthalene		
Bile[a]	67.2 ± 5.1	98	308 ± 21	99
Muscle[b]	2.25 ± 0.23	5.1 ± 0.4	1.25 ± 0.16	12.3 ± 0.9
Liver[b]	2.05 ± 0.12	8.5 ± 0.5	1.72 ± 0.01	24.0 ± 1.8
Blood[a]	1.83 ± 0.23		0.97 ± 0.08	
		2-methylnaphthalene		
Bile[a]	150 ± 24	96	1233 ± 201	100
Muscle[c]	4.9	2	2.6	10
Liver[c]	10.8	10	5.0	40
Blood[a]	3.3 ± 0.2		1.9 ± 0.1	
		1,2,4-trichlorobenzene		
Bile[a]	14.7 ± 0.8	65	87.5 ± 5.5	98
Muscle[c]	575[d]	0.8	299[d]	2.1
Liver[c]	22[d]	3.7	42[d]	6.2
Blood[a]	2.01 ± 0.12		1.03 ± 0.04	

Groups of eight trout were injected intraperitoneally with corn oil or a solution of NF in corn oil (100 mg/ml) at a rate of 1 ml/kg. After 48 h, groups of fish were exposed to one of the above chemicals for 24 h. The water levels of the chemicals for control and induced trout were naphthalene, 0.52 and 0.45 mg/liter; 2-methylnaphthalene, 0.28 and 0.36 mg/liter; and 1,2,4-trichlorobenzene, 0.20 and 0.20 mg/liter, respectively.

[a] Aliquots of blood and bile from each fish were used to determine levels of ^{14}C. Values are the average ± S.E. Metabolite determinations utilized pooled bile samples.

[b] Each sample consisted of pooled muscle or liver from two fish. Thus four samples per group were used to determine tissue ^{14}C levels and percentage of metabolites. Values are average ± S.E.

[c] Each sample consisted of pooled muscle or liver from all eight fish in the group.

[d] Tissue weights were not obtained. The total parent compound plus metabolites which were extracted is given.

control fish while ^{14}C levels in muscle, liver, and blood were about half those in control fish. The biliary ^{14}C from both groups of fish represented almost entirely polar material, while for liver and muscle the tissues from βNF-treated trout contained respectively 4 and 5 times the percentages of polar material found in these tissues from control trout.

The disposition of ^{14}C-1,2,4-trichlorobenzene also was studied in NF-injected and vehicle-injected trout. Biliary ^{14}C was 6 times as great in βNF-treated trout as in vehicle-injected fish. Levels of ^{14}C in liver also were great in NF-injected fish, while levels of ^{14}C in muscle and blood were lower. The percentages of ^{14}C present as polar material in bile, muscle, and liver were all higher in NF-treated trout than in vehicle-injected trout.

The bile from control and NF-induced fish exposed to each of the three ^{14}C-labeled chemicals was examined by TLC (Figure 51, Figure 52, and Figure 53). In all cases the bile from NF-treated fish had a greater polarity than from control trout.

Earlier it was shown that PBO, an inhibitor of xenobiotic metabolizing enzymes, affects the disposition and metabolism of ^{14}C-di-2-ethylhexylphthalate and ^{14}C-PCA in rainbow trout. The experiments demonstrate that pretreatment of trout with NF results in substantial increases in the amounts of biliary metabolites of the three ^{14}C-labeled chemicals. Substantial changes were observed in the tissue levels of ^{14}C accumulated from these chemicals and in the fraction of the ^{14}C that was present as metabolites.

Some aquatic pollutants, therefore, may be inducers or inhibitors of the activities of hepatic microsomal monooxygenase enzymes in fish, thereby affecting the metabolism and disposition of a variety of pollutants in these fish. Reports by Payne (1976) and Burns (1976) describe increased levels of microsomal enzyme activities which appear to result from environmental exposure to petroleum-derived pollutants. In order to evaluate whether such induction is significant, *in vivo* studies must be conducted on the disposition and metabolism of pollutants at the low levels that are found in the aquatic environment.

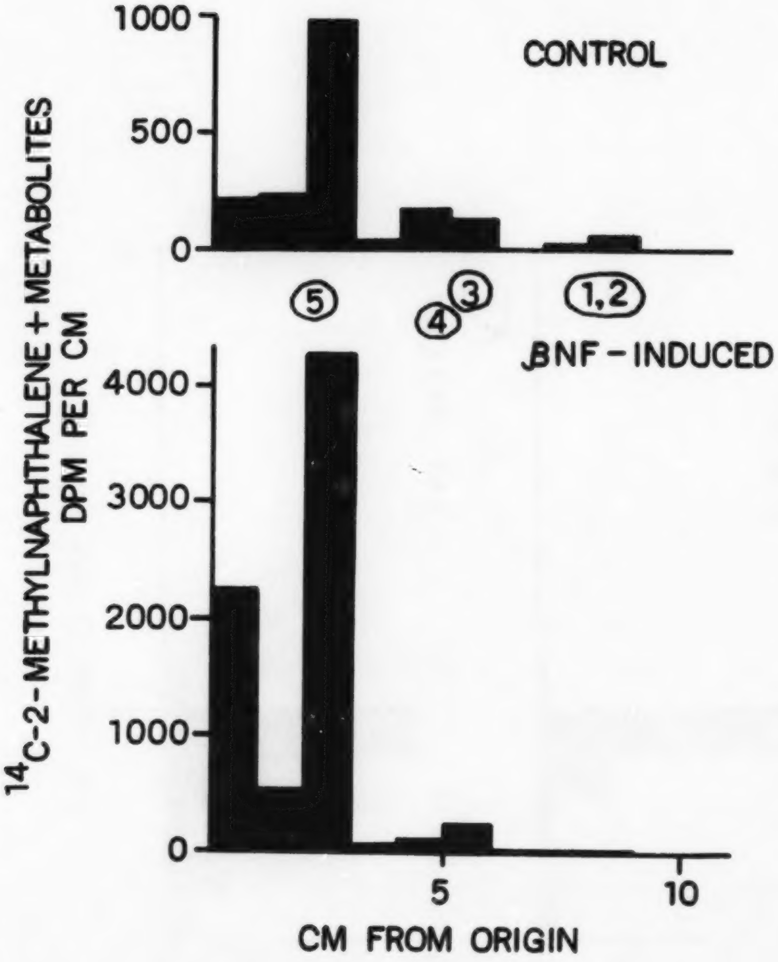

Figure 51. TLC of biliary ^{14}C from control and βNF-induced rainbow trout exposed to ^{14}C-2-methylnaphthalene for 24 h. The solvent used was the organic phase of butanol:NH_4OH:water (4:1:5). The circled numbers represent the mobility of standards where 1 = 2-methylnaphthalene, 2 = 2-methyl-1-naphthol, 3 = 1-naphthylsulfate, 4 = 1-naphthylglucopyranoside and 5 = 1-naphthylglucuronide.

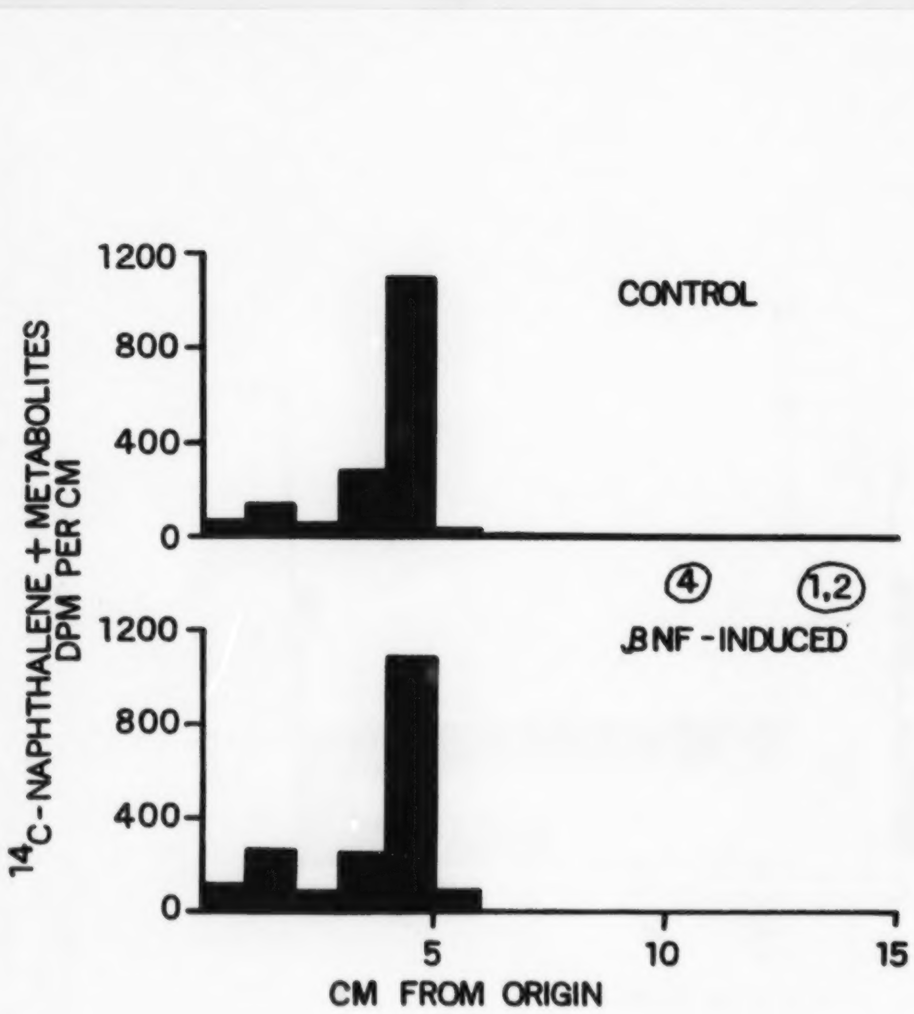

Figure 52. TLC of biliary ^{14}C from control and βNF-induced rainbow trout exposed to ^{14}C-naphthalene for 24 h.

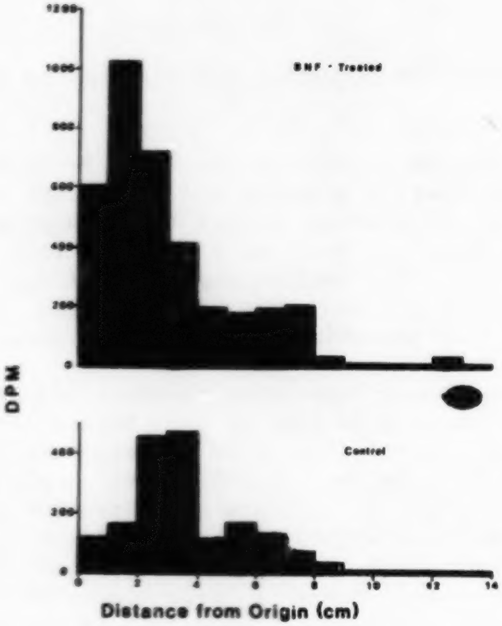

Figure 53. TLC of biliary ^{14}C from control and βNF-induced rainbow trout exposed to ^{14}C-1,2,4-trichlorobenzene for 24 h. The mobile phase was $CHCl_3$:MeOH:NH_4OH (8:4:1). The solid oval area represents the mobility of several trichlorophenols.

SECTION 5

SIGNIFICANCE AND POTENTIAL APPLICATIONS OF THE RESEARCH

The biotransformation of foreign chemicals by fish results in the presence of biotransformation products and the parent compound in fish tissues. The rate of elimination of such biotransformation products from fish tissues may be slower or more rapid than the rate of elimination of the parent chemicals. These observations suggest that fish tissues should be monitored for pollutants and their biotransformation products in order to evaluate the presence of potentially harmful substances.

The results have demonstrated that biotransformation products of certain chemicals accumulate in bile at much higher levels than that of the parent in water, even several days after the exposure was terminated. It is suggested that bile might be used for effective sampling in monitoring for environmental spills, etc. In the case of pentachlorophenol the feasibility of such monitoring was demonstrated by the results of a study concerning the contamination of a small lake near Hattiesburg, Mississippi with pentachlorophenol (Fate and Impact of Pentachlorophenol in a Freshwater Ecosystem by Richard H. Pierce, Jr., sponsored by the U.S.-EPA, Athens, Georgia). In that study, bile contained the highest level of pentachlorophenol and metabolites of any tissue analyzed. In the case of bass the bile level of pentachlorophenol and metabolites was thousands of times the water level of pentachlorophenol.

It has been demonstrated that certain chemicals can affect fish liver xenobiotic metabolizing enzymes and the biotransformation, disposition and elimination of other chemicals *in vivo*. Because of the wide variety of pollutants which reach the aqueous environment, interactions such as these should be anticipated from environmental pollutants.

REFERENCES

Adamson, R.H. 1967. Drug metabolism in marine vertebrates. Fed. Proc. 26:1047.

Adamson, R.H., R.L. Dixon, F.L. Francis, and D.P. Rall. 1965. Comparative biochemistry of drug metabolism by azo- and nitro-reductase. Proc. Nat. Acad. Sci. 54:1386.

Addison, R.F., M.E. Zinch, and D.E. Willis. 1977. Mixed function oxidase enzyme in trout (*Salvelinus fontinalis*) liver: Absence of induction following feeding of *P, p'*-DDT, or *p,p'*-DDE. Comp. Biochem. Physiol. 75C, 39.

Albro, P.W., and B. Moore. 1974. Identification of the metabolites of simple phthalate diesters in rat urine. Chromatog. 94:209.

Albro, B.W., R. Thomas, and L. Fishbein. 1973. Metabolism of diethylhexyl phthalate by rats: Isolation and characterization of the urinary metabolites. J. of Chrom. 76:32.

Alvares, A.P., D.R. Bickers, and A. Kappas. 1973. Polychlorinated biphenyls: A new type of inducer of cytochrome P-448 in the liver. Proc. Natl. Acad. Sci. U.S.A. 70:1321.

Alvares, A.P., and P. Siekevitz. 1973. Gel electrophoresis of partially purified cytochrome P-450 from liver of variously pretreated rats. Biochem. Biophys. Res. Communs. 54:923.

Ames, B.N. 1966. Assay of inorganic phosphate, total phosphate, and phosphatases. In: Methods in enzymology, I.S.P. Colowick and N.O. Kaplan, eds. Academic Press, New York. Vol. 8, p. 115.

Anders, M.W., and G.J. Mannering. 1966. Kinetics of the inhibition of the *N*-demethylation of ethylmorphine by 2-diethylaminoethyl 2,2-diphenyl valerate HCl (SKF-SF5A) and related compounds. Mol. Pharmacol. 2:319.

Anderson, J.W. 1975. Laboratory studies on the effects of oil on marine organisms: An overview. Am. Petroleum Inst. Publ. 4249:1.

Anderson, J.W., and J.M. Neff. 1975. Accumulation and release of petroleum hydrocarbons by edible marine organisms. In: International symposium on recent advances in the assessment of the health effects of environmental pollutants. 3:1461. (World Health Organization).

Augustinsson, K-B. 1959. Electrophoresis studies on bloodplasm esterases II. Avian, reptilian, amphibian and piscine plasmata. Acta Chem. Scand. 13:1089.

Bend, J.R., R.J. Pohl, and J.R. Fouts. 1973. Further studies of the microsomal mixed function oxidase system of the little shake, *Raja erinacea*, including its response to some xenobiotics. Bull. Mt. Desert Island Biol. Lab. 13:9.

Bhavnagary, H.M., and S.M. Ahmed. 1973. A spectrophotometric method for the micro-determination of piperonyl butoxide in the presence of pyrethrins. Analyst 98:792.

Bickers, D.R., J. Eisman, A. Kappas, and A.P. Alvares. 1975. Microscope immersion oils: Effects of skin application on cutaneous and hepatic drug-metabolizing enzymes. Biochem. Pharmacol. 24:779.

Boobis, A.R., O.W. Nebert, and J.S. Felton. 1977. Comparison of β-naphthaflavone and 3-methylcholanthrene as inducers of hepatic (cytachromets) P-448 and aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity. Mol. Pharmacol. 13:259.

Boylan, D.B., and B.W. Tripp. 1971. Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. Nature 230:44.

Branson, D.R., G.E. Blau, H.C. Alexander and W.B. Neely. 1975. Bioconcentration of 2,2'4,4'-tetrachlorobiphenyl in rainbow trout as measured by an accelerated test. Trans. Am. Fish. Soc. 104:785.

Brodie, B.B., and R.P. Maickel. 1962. Comparative biochemistry of drug metabolism. Proc. 1st International Pharmacol. Meeting 6:299-324.

Buhler, D.R. 1966. Hepatic drug metabolism in fishes. Fed. Proc. 25:343.

Buhler, D.R., and M.E. Rasmusson. 1968. The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25:223.

Burke, M.D., and R.T. Mayer. 1974. Ethoxyresorufin: Direct fluorimetric assay of a microsomal deethylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dis. 2:583.

Burns, K.A. 1976. Microsomal mixed function oxidases in an estuarine fish, *Fundulus heteroclitus*, and their induction as a result of environmental contamination. Comp. Biochem. Physiol. 53B:443.

Carlson, R.M. and R. Caple. 1976. Organo-chemical implications of water chlorination. In: The environmental impact of water chlorination. R.L. Jolly, ed. p. 73.

Carlson, R.M., and R. Caple. 1979. U.S. Environmental Protection Agency, Coal Leachate Project, R 803952-01-0, Final report.

Carter, J.E., D.B. Roll, and R.V. Petersen. 1974. The *in vitro* hydrolysis of di-(2-ethylhexyl)phthalate by rat tissues. Drug Metab. Dis. 2:341.

Casida, J.E., J.L. Engel, E.G. Essac, F.X. Kamienski, and S. Kuwatsuka. 1966. Methylene-C^{14}-dioxyphenyl compounds: Metabolism in relation to their synergistic action. Science 153:1130.

Cserjesi, A.J., and E.L. Johnson. Methylation of pentachlorphenol by *Trichoderma virgatum*. Can. J. Microbiol. 18:45.

Daniel, J.W., and H. Bratt. 1974. Absorption, metabolism and tissue distribution of di-(2-ethylhexyl) phthalate in rats. Toxicology 2:51.

deDuve, C., B.C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies b. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60:604.

DeFoe, D.L. 1975. A multichannel toxicant injection system for flow-through bioassays. J. Fish. Res. Bd. Can. 32:544.

Dent, J.G., C.R. Elcombe, K.J. Netter, and J.E. Gibson. 1978. Rat hepatic microsomal cytochrome(s) P-450 induced by polybrominated biphenyls. Drug. Metab. Dis., 6:96.

Dent, J.G., K.T. Netter and J.E. Gibson. 1976a. Effects of chronic administration of polybrominated biphenyls on parameters associated with hepatic drug metabolism. Res. Comm. Chem. Pathol. Pharmacol. 13:75.

Dent, J.G., K.J. Netter, and J.E. Gibson. 1976b. The induction of hepatic microsomal metabolism in rats following acute administration of a mixture of polybrominated biphenyls. Toxicol. Appl. Pharmacol. 38:237.

Dent, J.G., U. Roes, K.T. Netter, and J.E. Gibson. 1977. Stimulation of hepatic microsomal metabolism in mice by a mixture of polybrominated biphenyls. J. Tox. Environ. Health 6:637.

Dewaide, J.H. 1971. Metabolism of xenobiotics, Leijn, Numegen.

Dewaide, J.H., and P.T. Henderson. 1968. Hepatic *N*-dimethylation of aminopyrine in rat and trout. Biochem. Pharmacol. 17:901.

Dungs, W., and E. Beryheim-Irps. 1973. A new methylation method for the gas chromatography of barbituric acid. Anal. Lett. 6:185.

Dutton, G.J., and J.P. Montgomery. 1958. Glucuronide synthesis in fish and the influence of temperature. Biochem. J. 70:17P.

Ecobiochon, D.J., and A.M. Comeau. 1974. Comparative effects of commercial Aroclors on rat liver enzyme activities. Chem. Biol. Interactions 9:341.

Elcombe, C.E., and J.J. Lech. 1973. Induction of monooxygenation in rainbow trout by polybrominated biphenyls: A comparative study. Environ. Health Perspec. 23:309-314.

Elcombe, C.E., and J.J. Lech. 1979. Induction and characterization of hemoprotein(s) P-450 and monooxygenation in rainbow trout (*Salmo gairdneri*). Toxicol. Appl. Pharmacol. 49:437.

Estabrook, R.W., J. Peterson, J. Baron and A.G. Hildebrandt. 1972. The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. In: Methods in pharmacology. C.F. Chignell, ed. Appleton-Century-Crofts, New York. Vol. 2:303.

Fromm, P.O., and R.C. Hunter. 1969. Uptake of dieldrin by isolated perfused gills of rainbow trout. J. Fish. Res. Bd. Can. 26:1939.

Fukami, J., T. Mitsui, K. Fujunaga, and T. Shishido. 1969. The selective toxicity of rotenone between mammal, fish and insect. In: Biochemical Toxicology of Insecticides, Academic Press, R.D. O'Brien, and J. Yamamoto, eds. New York, p. 159.

Gianetto, R., and C. DeDuve. 1955. Tissue fractionation studies 4. Comparative study of the binding of acid phosphatase, -glucuronidase and cathepsin by rat-liver particles. Biochem. J. 59:433.

Glickman, A.H., C.N. Statham, A. Wu, and J.J. Lech. 1977. Studies on the uptake, metabolism, and disposition of pentachlorophenol and pentachloroanisole in rainbow trout. Toxicol. and Appl. Pharmacol. 41:649.

Goldstein, J.A., P. Hickman, V.W. Burse, and H. Bergman. 1975. A comparative study of two polychlorinated biphenyl mixtures (Aroclors 1242 and 1016) containing 42% chlorine on induction of hepatic porphyria and drug metabolizing enzymes. Toxicol. Appl. Pharmacol. 32:461.

Goldstein, J.A., P. Hickman, H. Bergman, J.D. McKinney, and M.P. Walker. 1977. Separation of pure polychlorinated biphenyl isomers into two types of inducers on the basis of induction of cytochrome P-450 or P-448. Chem. Biol. Interactions 17:69.

Green, D.E., S. Mii, and P.M. Kohout. 1955. Studies on the terminal electron transport system 1. Succinic dehydrogenase. J. Biol. Chem. 217:551.

Guenthner, T.M., and G.J. Mannering. 1977. Induction of hepatic monooxygenase systems of pregnant rats with phenobarbital and 3-methylcholanthrene. Biochem. Pharmocol. 26:567.

Gutenmann, W.H., and D.J. Lisk. 1965. Conversion of 4-(2,4-DB) herbicide to 2,4-D by bluegills. New York Fish Game J. 12:108.

Hamelink, J.L., R.C. Waybrant, and R.C. Ball. 1971. A proposal: Exchange equilibria control the degree chlorinated hydrocarbons are biologically magnified in lentic environments. Trans. Am. Fish. Soc. 100:207.

Hansen, A.R., and J.R. Fouts. 1972. Some problems in Michaelis-Manten kinetic analysis of benzpyrene hydroxylase in hepatic microsomes from polycyclic hydrocarbon-pretreated animals. Chem. Biol. Interactions 5:167.

Hanson, L.G., I.P. Kapoor, and R.L. Metcalf. 1972. Biochemistry of selective toxicity and biodegradability: Comparative *O*-dealkylation by aquatic organisms. Comp. Gen. Pharmacol. 3:339.

Hites, R.A., and K. Biemann. 1972. Water pollution: Organic compounds in the Charles River, Boston. Science 178:158.

Hogan, J.W., and C.O. Knowles. 1972. Metabolism of diazinon by fish liver microsomes. Bull. Environ. Contam. Toxicol. 8:61.

Hodgson, E., and R. M. Philpot. 1974. Interaction of methylenedioxyphenyl (1,3-benzodioxle) compounds with enzymes and their effect on mammals. Drug Metab. Rev. 3:231-301.

Huang, K.C., and F.F. Collins. 1962. Conjugation and excretion of aminobenzoic acid isomers in marine fishes. J. Cell. Comp. Physiol. 60:29.

Huang, Y.C., and R.G. Kemp. 1971. Properties of a phosphodiesterase with high affinity for adenosine 3',5'-cyclic phosphate. Biochem. 10:2278.

Hubscher, G., and G.R. West. 1965. Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. Nature 205:799.

Hunn, J.B., R.A. Schoettger, and W.A. Willford. 1968. Turnover and urinary excretion of free and acetylated M.S. 222 by rainbow trout, *Salmo gairdneri*. J. Fish Res. Bd. Can. 25:25.

Imai, Y., and R. Sato. 1966. Evidence for two forms of P-450 hemoprotein in microsomal membranes. Biochem. Biophys. Res. Commun. 23:5.

James, M.O., J.R. Fouts, and J.R. Bend. 1977. Xenobiotic metabolizing enzymes in marine fish. In: Pesticides in aquatic environments, Plenum Press, New York. p. 171.

Jolly, R.L., G. Jones, W.W. Pitt, and J.E. Thompson. 1976. Chlorination of organics in cooling waters and process effluents. In: The environmental impact of water chlorination. R.L. Jolly, ed. 115 p.

Kamienski, F.X., and S.D. Murphy. 1971. Biphasic effects of methylenedioxphenyl synergists on the action of hexobarbital and organophosphate insecticides in mice. Toxicol. Appl. Pharmacol. 18:833.

Laemmli, U.K, and M. Favre. 1973. Maturation of the head of bacteriophage T4.I DNA packaging event. J. Mol. Biol. 80:575.

Larson, R.A., and J.C. Weston. 1976. Analysis of water extracts of crude petroleum by gel permeation chromatography. Bull. Environ. Contam. Toxicol. 16:44.

Lech, J.J., and C.N. Statham. 1975. Role of gluronide formation in the selective toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) for the sea lamprey: Compariative aspects of TFM uptake and conjugation in sea lamprey and rainbow trout. Toxicol. Appl. Pharmacol. 31:150.

Lee, C.C., W.K. Craig, and P.J. Smith. 1974. Water-soluble hydrocarbons from crude oil. Bull. Environ. Contam. Toxicol. 12:212.

Lee, R.F., R. Sauerheber, and G.H. Dobbs. 1972. Uptake, metabolism, and discharge of polycyclic aromatic hydrocarbons by marine fish. Mar. Biol. 17:201.

Levvy, G.A. 1952. The preparation and properties of β-glucuronidase 4. Inhibition by sugar acids and their lactones. Biochem. J. 52:464.

Ljungquist, A., and K-B. Augustinsson. 1971. Purification and properties of two carboxylesterases from rat-liver microsomes. Eur. J. Biochem. 23:303.

Lotliker, P., E.C. Miller, J.A. Miller, and J.E. Halver. 1967. Metabolism of the carcinogen 2-acetylaminofluorene by rainbow trout. Proc. Soc. Exp. Biol. Med. 124:160.

Macek, K.J., M.E. Barrow, R.F. Frasny, and B.H. Sleight, III. 1975. Bioconcentration of ^{14}C-pesticides by bluegill sunfish during continuous aqueous exposure. In: Structure-Activity Correlations in Studies of Toxicity and Bioconcentration with Aquatic Organisms. G.D. Veith and D.E. Konasewich, eds. p. 119.

Maren, T.H., R. Embry, and L.E. Broder. 1968. The excretion of drugs across the gill of the dogfish, *Squalus acanthias*. Comp. Biochem. Physiol. 26:853.

Melancon, M.J. Jr., and J.J. Lech. 1976. Distribution and biliary excretion products of di-2-ethylhexyl phthalate in rainbow trout. Drug Metab. Dis. 4:112.

Melancon, M.J. Jr., and J.J. Lech. 1977. Metabolism of di-2-ethylhexyl phthalate by subcellular fractions from rainbow trout liver. Drug Metab. Dis. 5:29.

Melancon, M.J. Jr., and J.J. Lech. 1978. Distribution and elimination of napthalene and 2-methylnapthalene in rainbow trout during short- and long-term exposures. Arch. Environ. Contam. Toxicol. 7:207-220.

Melancon, M.J. Jr., and J.J. Lech. 1979. Structural requirements for the inhibition of phthalate ester hydrolysis in rainbow trout by methylenediosyphenyl compounds. Xenobiotica 9:317.

Melancon, M.J. Jr., and J.J. Lech. 1979. Uptake, biotransformation, disposition, and elimination of 2-methylnaphthalene and naphthalene in several fish species. In: Aquatic Toxicology, ASTM STP 667, L.L. Marking and R.A. Kimerle, eds. Am. Soc. for Testing and Materials.

Melancon, M.J. Jr., J. Saybolt, and J.J. Lech. 1977. Effect of piperonyl butoxide on disposition of di-2-ethylhexyl phthalate by rainbow trout. Xenobiotica 7:633.

Montesano, R., A.J. Ingram, and P.N. Magee. 1973. Metabolism of dimethylnitrosamine by amphibians and fish *in vitro*. Experientia 29:599.

Moore, R.W., and S.D. Aust. 1977. Induction of drug metabolizing enzymes by 2,2', 4,4', 5,5'-hexabromobiphenyl. Pharmacol. 19:162.

Moore, R.W., S.D. Sleight, and S.D. Aust. 1978. Induction of liver microsomal drug metabolizing enzymes by 2,2', 4,4', 5,5'-hexabromobiphenyl. Toxicol. Appl. Pharmacol. 44(2):309-321.

Mount, D.I., and W.A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Res. 1:21.

Murphy, S.D. 1966. Liver metabolism and toxicity of thiophosphate insecticides in mammalian, avian and piscine species. Proc. Soc. Exp. Biol. Med. 123:392.

Nakatsugawa, T., and P.A. Dahm. 1967. Microsomal metabolism and parathion. Biochem. Pharmacol. 16:25.

Nakatsugawa, T., N.M. Tolman, and P.A. Dahm. 1969. Oxidative degradation of diazinon by rat liver microsomes. Biochem. Pharmacol. 18:685.

Nazir, D.J., A.P. Alcaraz, B.A. Bierl, M. Beroza, and P.P. Nair. 1971. Isolation, identification, and specific localization of di-2-ethylhexyl phthalate in bovine heart muscle mitochondria. Biochem. 10:4228.

Neely, W.B., D.R. Branson, and G.E. Blau. 1974. Partition coefficient to measure bioconcentration potential of organic chemicals in fish. Environ. Sci. Technol. 8:1113.

Neff, J.M., B.A. Cox, D. Dixit, and J.W. Anderson. 1976. Accumulation and release of petroleum-derived aromatic hydrocarbons by four species of marine animals. Marine Biology 38:279.

Olson, L.E., J.L. Allen, and J.W. Hogan. 1977. Biotransformation and elimination of the herbicide, dinitramine, in carp. J. Agr. Food Chem. 25(3):554-556.

Payne, J.F. 1975. Field evaluation of benzopyrene hydroxylase induction as a monitor of marine petroleum pollution. Science 191:945.

Payne, J.F., and W.R. Penrose. 1975. Induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase in fish by petroleum. Bull. Environ. Contam. Toxicol. 14:112.

Pederson, M.G., W.K. Hershberger, P.K. Zachariah, and M.R. Juchau. 1976. Hepatic biotransformation of environmental xenobiotics in six strains of rainbow trout (*Salmo gairdneri*). J. Fish Res. Bd. Can. 33:66.

Potter, J.L., and R.D. O'Brien. 1964. Parathion activation by livers of aquatic and terrestial vertebrates. Science 144:55.

Pohl, R.J., J.R. Bend, A.M. Guarino, and J.R. Fouts. 1974. Hepatic microsomal mixed-function oxidase activity of several marine species from coastal Maine. Drug Metab. Dis. 2:545.

Poland, A., and E. Glover. 1977. Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase: A study of the structure activity relationship. Mol. Pharmacol. 13:924.

Rodgers, C.A., and D.L. Stalling. 1972. Dynamics of an ester of 2,4-D in organs of three fish species. Weed Sci. 20:101.

Ross, E., and G. Schatz. 1973. Assay of protein in the presence of high concentrations of sulfhydryl compounds. Analyt. Biochem. 54:304.

Roubal, W.T., T.K. Collier, and D.C. Malins. 1977. Accumulation and metabolism of carbon-14 labeled benzene, naphthalene, and anthracene by young coho salmon (*Oncorhynchus kistuch*). Arch. Environ. Contam. Toxicol. 5:513-529.

Sanborn, H.R., and D.C. Malins. 1977. Toxicity and metabolism of naphthalene: A Study with marine larval invertebrates. Proc. Soc. Exp. Biol. Med. 154:151.

Sanborn, J.R., R.L. Metcalf, C-C. Yu, and P-Y. Lu. 1975. Plasticizers in the environment: The fate of di-*N*-octylphthalate (DOP) in two model ecosystems and uptake and metabolism of DOP by aquatic organisms. Arch. Environ. Contam. Toxicol. 3:244.

Schenkman, J.D., H. Remmer, and R.W. Estabrook. 1967. Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3:113-123.

Schoettger, R.A. 1970. Toxicology of thiadan in several fish and aquatic invertebrates. Inv. Fish Control 35:3.

Schultz, D.P. 1973. Dynamics of a salt of (2,4-dichlorophenoxy) acetic acid in fish, water, and hydrosol. J. Agr. Food Chem. 21:185.

Schwark, W.S., and D.J. Ecobichon. 1968. Subcellular localization and drug-induced changes of rat liver and kidney esterases. J. Physiol. Pharmacol. 46:207.

Shibko, S., and A.L. Tappel. 1964. Distribution of esterases in rat liver. Arch. Biochem. Biophys. 106:259.

Sladek, N.E., and G.J. Mannering. 1966. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun. 24:668.

Solyom, A., and E.G. Trams. 1972. Enzyme markers in characterization of isolated plasma membranes. Enzyme 13:329.

Sottocasa, G.L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. J. Cell. Bio. 32:415.

Stalling, D.L., J.W. Hogan, and J.L. Johnson. 1973. Phthalate ester residues - their metabolism and analysis in fish. Envir. Health Perspec. 3:159.

Stanton, R.H., and M.A.Q. Khan. 1973. Mixed-function oxidase activity toward cyclodiene insecticides in bass and bluegill sunfish. Pest. Biochem. and Physiol. 3:354.

Statham, C.N., C.R. Elcombe, S.P. Szyjka, and J.J. Lech. 1978. Effect of polycyclic aromatic hydrocarbons on hepatic microsomal enzymes and disposition of methylnaphthalene in rainbow trout *in vivo*. Xenobiotica 8:65-71.

Statham, C.N., and J.J. Lech. 1975. Metabolism of 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73) in rainbow trout. J. Fish Res. Bd. Can. 32:515.

Statham, C.N., and J.J. Lech. 1976. Studies on the mechanism of potentiation of the acute toxicity of 2,4-D *N*-butylester and 2',5-dichloro-4'-nitrosalicylanilide in rainbow trout by carbaryl. Toxicol. Appl. Pharmacol. 36:281.

Statham, C.N., M.J. Melancon, Jr., and J.J. Lech. 1976. Bioconcentration of xenobiotics in trout bile: A proposed monitoring aid for some waterborne chemicals. Science 193:680.

Statham, C.N., S.P. Szyjka, L.A. Menahan, and J.J. Lech. 1977. Fractionation and subcellular localization of marker enzymes in rainbow trout liver. Biochem. Pharmacol. 26:1395.

Stegeman, J., R.L. Binder, and A.M. Orren. 1980. Hepatic and extrahepatic microsomal electron transport components and mixed function oxidases in the marine fish *Stenotomus bersicolor*. Biochem. Pharmacol.

Sweeley, C.C., W.H. Elliott, I. Fries, and R. Ryhage. 1966. Mass spectrometric determination of unresolved components in gas chromatographic effluents. Anal. Chem. 38:1549.

Tanaka, A., T. Adachi, T. Takahashi, and T. Yamaha. 1975. Biochemical studies of phthalic esters I. Elimination, distribution and metabolism of di-(2-ethylhexyl)phthalate in rats. Toxic. 4:253.

Ullrich, V., and P. Weber. 1972. The *O*-dealkylation of 7-ethoxycoumarin by liver microsomes. Hoppe-Seyler's Z. Physiol. Chem. 353:1171.

Underhay, E., S.J. Holt, H. Beaufay, and C. deDuve. 1956. Introcellular localization of esterase in rat liver. J. Biophys. Biochem. Cytol. 2:635.

Wiebel, F.J., J.C. Leutz, L. Diamond, and H.V. Gelboin. 1971. Aryl hydrocarbon (benzo[a]pyrene) hydroxylase in microsomes from rat tissues: Differential inhibition and stimulation by benzoflavone and organic solvents. Arch. Biochem. Biophys. 144:78.

Williams, D.T. 1973. Dibutyl- and di-(2-ethylhexyl)phthalate in fish. Agr. Food Chem. 21:1128.

Williams, D.T., and B.J. Blanchfield. 1974. Retention, excretion and metabolism of di-(2-ethylhexyl)phthalate administered orally to the rat. Bull. Environ. Contam. Toxicol. 11:371.

Williams, D.T., and B.J. Blanchfield. 1975. The retention , distribution, excretion, and metabolism of dibutyl phthalate-7-^{14}C in the rat. Agr. Food Chem. 23:854.

Yamamoto, I. 1970. Mode of action of pyrethroids, nicotinoids, and rotenoids. Ann. Rev. Entomol. 15:257.

Yobs, A. R. 1972. Levels of polychlorinated biphenyls in adipose tissue of the general population of the nation. Environ. Health Perspec. 1:79.

Zitko, V. 1977. The accumulation of polybrominated biphenyl by fish. Bull. Environ. Contam. Toxicol. 17:285.